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Fatty Acid Composition of Tissue Cholesterol Esters in Elderly Humans with Atherosclerosis.* (25624)

LEON SWELL, HENRY FIELD, JR., P. E. SCHOOLS, JR., AND C. R. TREADWELL
Vet. Admin. Center, Martinsburg, W. Va., and Dept. of Biochemistry, School of Medicine, George Washington University, Washington, D. C.

The current interest in the fatty acid composition of the cholesterol esters in human tissues is due to the suggested role of cholesterol esters in essential fatty acid transport and metabolism(1) and the hypothesis that in atherosclerosis there is a replacement of unsaturated fatty acids by saturated or non-essential unsaturated fatty acid in tissue cholesterol esters(2). In several recent studies (3-7) a comparison of serum and plaque cholesterol ester fatty acid (CEFA) was made in relation to atherosclerosis. In the summary of these studies(6) it was concluded that "at present the evidence is too discrepant to lend support to a theory of atherogenesis based specifically on differences in CEFA in plasma and atheroma." These investigators used the alkali isomerization technic to measure polyunsaturated fatty acids with saturated and monoenoic acids being obtained by difference or by gas-liquid chromatography. While gas-liquid chromatography yields more definitive data, the published studies(4,7) so far have described limited groups or pooled samples, or separation of saturated and unsaturated fatty acids of the same chain length was not achieved. A recent study(8) on deposition and turnover of cholesterol, using tracer doses of cholesterol-4-C^{14} in elderly male humans, indicated that a large portion of the cholesterol in the normal intima and thickened intima (early plaques) of the aorta was derived from the plasma. The present study was undertaken to obtain further information concerning the nature of cholesterol deposition in atherosclerosis. The CEFA of

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TABLE I. Retention Volumes of Fatty Acid Methyl Esters Relative to Methyl Myristate.

Fatty acid*		Apparent retention vol;† methyl myristate = 1.00
Chain length carbons	No. double bonds	
8	0	.20
10	0	.35
12	0	.58
12	1	.74
14	0	1.00
14	1	1.23
16	0	1.68
16	1	2.00
16	2	2.55
18	0	2.81
18	1	3.29
18	2	4.13
20	0	4.82
18	3	5.43
22	0	8.01
20	4	9.20

* Stationary phase was succinate polyester of diethylene glycol; temp. 209°C; 2 M column; helium flow rate 82 ml/min.

† Fatty acids listed in order of elution; calculated from retention time (peak emergence time relative to air peak).

the free-lying plaque material of advanced atheromatous lesions was compared with those of the media and liver of the original subjects and with the serum of 6 patients with occlusive atherosclerosis who were in good nutritional status.

Methods and materials. The tissues, other than serum, were obtained at autopsy from 6 hospitalized male patients (aged 61-74 years). Five died of chronic disease and one died suddenly of a recurrent acute myocardial infarction. The subjects were in good nutritional status except for a short terminal period. Autopsies were performed within 6 hours after death and portions of the abdominal aorta and liver obtained. The aorta was freed of adherent tissue and its layers carefully dissected along their cleavage planes into adventitia, media and intima. The intima were partitioned, as far as their occurrence permitted, into normal appearing portions, thickened portions or plaques, and free-lying lipid containing material (either solid or granular) from far-advanced plaques. Data on the aorta reported here are on the media and the free-lying lipid material. Fasting serum was obtained from 6 elderly male patients with occlusive atherosclerosis, in good nutritional status. Lateral roentgenograms of the abdomen were obtained for evaluation of degree of atherosclerosis by observation of extent of calcification in the abdominal aorta. Lipid extracts of the tissues and serum were prepared as described earlier(9). Cholesterol esters were separated from other lipid components by chromatography on silicic acid (10). Quantitative recovery of sterol esters from the silicic acid column in the 1% ethyl ether-petroleum ether fraction was verified with the aid of labeled cholesterol-4-C^{14} oleate. No overlap with the triglyceride fraction was noted as checked with triolein-1-C^{14}. The isolated cholesterol esters were interesterified in HCl-methanol and the methyl esters sublimed according to the method of Stoffel *et al.* (11). Gas-liquid chromatography was carried out in a Perkin-Elmer Model 154-C Vapor Fractometer using a hot wire detector. The column, 2 M in length, was maintained at a constant temperature of 209°C. The carrier gas was helium; a flow rate of 82 ml/min was used for elution. The inert support was celite 545 and the stationary phase was a succinate polyester of diethylene glycol(12). With this stationary phase it was possible to separate the individual components of mixtures of saturated and unsaturated fatty acids ranging from C_6 to C_{20} within 30 minutes. The apparent retention volumes relative to methyl myristate are shown in Table I. Methyl myristate had a retention time of 3.1 minutes relative to air peak. The areas under the peaks were obtained by triangulation and fatty acid composition calculated from those values(13). Table II shows recovery of a mixture of pure methyl esters.

Results. Plaque material (Table III).

TABLE II. Analysis of a Standard Mixture of Fatty Acid Methyl Esters.

Methyl ester	Weighed, mg	Found, mg	Recovery, %
Laurate	20	21.3	106.5
Myristate	50	47.7	95.4
Palmitate	100	104.7	104.7
Stearate	100	101.9	101.9
Oleate	100	95.3	95.3
Linolenate	100	96.4	96.4
Erucate	100	95.3	95.3
Total	570	562.6	98.7

TABLE III. Cholesterol Ester Fatty Acid Composition of Isolated Lipid Material from Far-Advanced Atherosclerotic Plaques in Elderly Subjects.

Fatty acid* Chain length carbons	No. double bonds	% of fatty acids in subjects 1	2	3	4	5	6	Avg
6 to 12		1.3	.8	.3	1.0	.7	1.3	.9 ± .4†
14	0	.7	1.5	.6	.3	.3	1.0	.7 ± .5
14	1	1.3	.3	.3	.2	.2	.3	.4 ± .4
16	0	26.4	25.2	16.0	36.0	19.2	27.7	25.1 ± 7.0
16	1	5.2	8.8	7.3	1.5	8.0	10.5	6.7 ± 3.2
16	2	.2	.3	.4	.2	.4	.4	.3 ± .1
18	0	.2	.7	.7	.5	.6	1.5	.8 ± .4
18	1	39.5	39.5	36.0	36.0	37.2	37.7	37.8 ± 1.6
18	2	21.9	22.3	32.7	23.4	32.7	16.9	25.1 ± 6.4
18	3	.9	.3	.5	.3	.2	.4	.4 ± .2
20	4	2.4	.3	5.2	.6	.5	1.3	1.8 ± 1.9

* These represent major acids found; very small amounts of 18:4, 20:0, 20:3 and others were also detected. Values were calculated as % of total fatty acids determined.

† Stand. dev.

TABLE IV. Cholesterol Ester Fatty Acid Composition of Aortic Media in Elderly Subjects.

Fatty acid* Chain length carbons	No. double bonds	% of fatty acids in subjects 1	2	3	4	5	6	Avg
6 to 12		1.3	2.8	2.7	1.8	2.8	2.1	2.3 ± .6
14	0	.9	1.7	1.5	1.8	1.6	1.7	1.5 ± .3
14	1	.4	.5	1.1	.4	.5	.6	.6 ± .3
16	0	16.1	23.4	18.3	25.8	17.7	15.1	19.4 ± 4.4
16	1	2.8	5.1	3.1	5.9	3.5	1.7	3.7 ± 1.5
16	2	.1	.1	.3	.3	.2	.1	.2 ± .1
18	0	.1	.5	.9	.3	.4	.6	.5 ± .3
18	1	25.9	32.6	23.0	31.0	21.0	28.0	26.9 ± 2.9
18	2	45.3	28.2	40.7	30.5	44.6	47.3	39.4 ± 8.1
18	3	1.3	.2	.3	.4	.2	.3	.5 ± .4
20	4	5.8	4.9	8.1	1.8	7.5	2.5	5.1 ± 2.2

* See footnotes to Table III.

Three fatty acids[†] (palmitic, oleic and linoleic) comprised 88% of total CEFA of the far-advanced plaques. The major acid was oleic acid and total monoenoic fatty acids accounted for 45% of the CEFA. The polyunsaturated fatty acids (linoleic, linolenic and arachidonic) comprised only 27% of total fatty acids with linoleic acid the major acid in this group. Arachidonic acid was low in plaque material. Small amounts of short chain acids and stearic acid were detected in all samples.

Media (Table IV). As with plaque material, major CEFA's of the aortic media were palmitic, oleic and linoleic (86% of the total), with linoleic present in greatest amount (39% of the total). The monoenoic fraction accounted for 31% of total fatty acids and polyunsaturated fatty acids 45%. Short chain fatty acids accounted for more than 4% of the total fatty acids. Very little stearic acid was present.

Liver (Table V). In liver, polyunsaturated fatty acids made up only 20% of CEFA. The major acid was oleic acid (37%); monoenoics accounted for 45% of the fatty acids. A considerable amount of stearic acid was also present (8.4%) and saturated acids constituted 35% of total acids. Short chain fatty acids accounted for 7% of total fatty acids.

Serum (Table VI). In serum the major CEFA's were linoleic acid (43.3%), oleic acid (25.8%), and palmitic acid (13.7%). Polyunsaturated fatty acids comprised 52% of total fatty acids. Saturated fatty acids ac-

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[†] For simplicity the acids designated in Tables by chainlength and degree of saturation are referred to in the text by their common names.

TABLE V. Cholesterol Ester Fatty Acid Composition of Livers in Elderly Subjects.

Fatty acid* Chain length carbons	No. double bonds	% of fatty acids in subjects 1	2	3	4	5	6	Avg
6 to 12		.8	1.5	3.7	2.9	3.4	2.9	2.5 ± 1.1
14	0	1.3	1.2	5.9	2.5	3.1	2.9	2.8 ± 1.7
14	1	.5	.9	4.6	1.3	1.9	2.0	1.9 ± 1.3
16	0	24.0	36.3	25.9	16.5	23.1	18.8	24.1 ± 7.7
16	1	3.6	3.9	10.7	2.9	6.6	5.3	5.5 ± 2.9
18	0	8.4	6.0	5.9	9.7	10.3	9.9	8.4 ± 1.6
18	1	40.1	36.5	38.1	35.7	33.8	39.6	37.3 ± 2.4
18	2	17.2	10.8	3.9	26.0	12.8	27.0	16.3 ± 9.1
18	3	.2	.3	Tr	Tr	Tr	Tr	.2 ± .2
20	4	3.7	2.4	1.5	2.5	5.0	2.9	3.0 ± 1.2

* See footnotes to Table III.

counted for only about 16% of the total. Short chain fatty acids were also detected. In all sera studied small amounts of C_{20} saturated and C_{20} trienoic acids were also found.

Discussion. For evaluation and comparison of our data with those in the literature (3-7), the following points are significant. (a) Data on the portions of the aorta involved a careful separation by dissection and analysis of the different portions from each individual. (b) Average values were derived from separate analyses of individual specimens. Consequently, as presented, the data provide information on variability of CEFA between several subjects. The consistency of CEFA values in each group of determinations is noteworthy. (c) Serum data were from a separate series of patients with occlusive atherosclerosis, of the same age and sex as the subjects from which the tissues were obtained, and in good nutritional status at time blood samples were obtained. This eliminated possible changes in blood composition due to poor nutrition immediately prior to death.

There were striking differences between CEFA composition of the free-lying plaque material and the serum (Table VI). Plaque material had significantly ($P<.01$) greater amounts of palmitic and monoenoic acids (chiefly oleic with smaller amounts of palmitoleic) and significantly less ($P<.01$) polyunsaturated fatty acids (linoleic, arachidonic) than the serum.

The differences between CEFA compositions of plaque material and of those of the media were the same as those differences between plaque material and serum. CEFA compositions of serum and of media had a very notable similarity. This suggests that cholesterol esters were deposited in the media

TABLE VI. Comparison of the Cholesterol Ester Fatty Acid Composition of Tissues and Serum from Elderly Subjects.

Fatty acid Chain length carbons	No. double bonds	% of fatty acids in subjects (avg) Plaque material	Media	Liver	Serum*
6 to 12		.9 ± .4	2.3 ± .6	2.5 ± 1.1	1.0 ± .2
14	0	.7 ± .5	1.5 ± .3	2.8 ± 1.7	.9 ± .2
14	1	.4 ± .4	.6 ± .3	1.9 ± 1.3	.3 ± .1
16	0	25.1 ± 7.0	19.4 ± 4.4	24.1 ± 7.7	13.7 ± 1.4
16	1	6.7 ± 3.2	3.7 ± 1.5	5.5 ± 2.9	4.1 ± 1.1
16	2	.3 ± .1	.2 ± .1	Tr	.4 ± .1
18	0	.8 ± .4	.5 ± .3	8.4 ± 1.6	1.3 ± .3
18	1	37.8 ± 1.6	26.9 ± 2.9	37.3 ± 2.4	25.8 ± 3.5
18	2	25.1 ± 6.4	39.4 ± 8.1	16.3 ± 9.1	43.3 ± 4.7
18	3	.4 ± .2	.5 ± .4	.2 ± .2	.7 ± .4
20	0				.7 ± .4
20	3				.3 ± .2
20	4	1.8 ± 1.9	5.1 ± 2.2	3.0 ± 1.2	7.5 ± 2.2

* Values are avg for 6 subjects.

by diffusion, in contrast to the plaque material.

In the liver, a larger portion of the CEFA were monenoics and saturated fatty acids and a smaller portion were polyunsaturated acids than were found in the serum. These findings agree with those of a study of turnover and synthesis of cholesterol, using single tracer doses of cholesterol-4-C^{14}, which indicated that synthesis of cholesterol esters in the liver was much too slow to account for all of the esters of the plasma(8). The differences between CEFA of liver and of plasma might be due to a selectivity of synthesis in, or of release from the liver, of certain cholesterol esters. One or both could explain the very high content of arachidonic acid in CEFA of serum of rats.[‡]

This study does not support the concept of indiscriminate deposition of blood cholesterol esters in the intima of the aorta(4). Data of the present study indicate that a specificity of deposition or synthesis in different tissues exist, since the tissues studied differed in CEFA composition, except serum and media.

These results offer some support for Sinclair's hypothesis(2) insofar as there was found a significantly larger amount of saturated and monoenoic acids in the CEFA of isolated plaque material than in those of serum. They do not provide information on the mechanism thereof. Degree of atherosclerosis, as evaluated by extent of calcification of the abdominal aorta demonstrable in lateral roentgenograms of the abdomen, had no correlation with the arachidonic or linoleic acid content in CEFA of the serum. That negative finding is of doubtful significance. Composition of CEFA of the serum now does not necessarily indicate what it was during the approximately 50 years in which atherosclerosis was progressing.

Summary. The fatty acids esterified with cholesterol have been determined by gas-liquid chromatography in the media, free-lying lipid containing material of far-advanced plaques, and livers obtained at autopsy from 6 atherosclerotic subjects. For comparison, serum CEFA in 6 well-nourished subjects with occlusive atherosclerosis was also determined. Of tissues studied, composition of CEFA of the aortic media was very similar to that of serum. Plaque material contained a greater proportion of saturated and monoenoic fatty acids than either media or serum. Liver CEFA contained considerably less polyunsaturated fatty acids and more saturated and monoenoic acids than did serum or media. The major CEFA of media and serum was linoleic acid and major fatty acid of plaque material and liver was oleic acid. The significance of these findings in relation to the role of unsaturated fatty acids in atherogenesis is discussed.

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[‡] Unpublished data.

Swelling of Liver Mitochondria from Rats Fed Diets Deficient in Essential Fatty Acids.* (25625)

Takuya Hayashida and Oscar W. Portman† (Introduced by F. J. Stare)
Dept. of Nutrition, Harvard School of Public Health, Boston, Mass.

Liver mitochondria from rats fed diets deficient in essential fatty acids (EFA)‡ have been shown to uncouple oxidative phosphorylation(1); subsequently, Levin *et al.*(2) demonstrated that normal rat liver mitochondria resemble those from rats deficient in EFA with respect to succinate oxidizing capacity and oxidative phosphorylation when studied in a suspending medium of low osmolarity. These workers also showed that liver mitochondria from rats fed diets deficient in EFA were slightly larger than normal mitochondria, were less opaque and contained many crescent-shaped components. Furthermore, increased fragility of the red blood cells as well as increased permeability of the skin of rats has been demonstrated(3) in EFA deficiency. Such studies suggest that both the cellular and mitochondrial membrane of the rat fed diets deficient in essential fatty acids differ morphologically from those of normal rats. On the other hand, since the early observations by Cleland(4) and by Raaflaub (5), swelling phenomena have been frequently used to study structural alterations of mitochondria in relation to biochemical properties. This communication describes studies which show that liver mitochondria from rats deficient in EFA have characteristic patterns of swelling which are different in several ways from those of normal rats. The fatty acid composition of the mitochondrial, microsomal and supernatant fractions isolated from livers of rats fed diets deficient in essential fatty acids and from normal rats are also presented.

Methods. Weanling male albino rats housed in individual cages were divided into groups and maintained on experimental diets (6) *ad libitum* for 15 to 19 weeks. One group of rats was fed a purified diet including 5% by weight of corn oil; the other group received a diet devoid of fat. Liver mitochondria were isolated in 0.88 M sucrose by the method of Ziegler *et al.*(7). Glass-redistilled water and a glass-Teflon homogenizer were used throughout this study. One ml of the resulting mitochondrial preparation was equivalent to 1.0 g of fresh rat liver. The swelling of mitochondria was measured spectrophotometrically by the method of Lehninger *et al.*(8) as slightly modified. The standard test system was 3.0 ml of 0.30 M sucrose—0.02 M Tris buffer, pH 7.4, using a Beckman DU Spectrophotometer equipped with thermospacers to maintain temperature in the cuvette carriage chamber at 23-24°C. Studies of mitochondrial swelling under various experimental conditions were performed on liver mitochondria from 12 control and 12 rats deficient in EFA. Aliquots of the liver mitochondrial preparations, as well as microsomal and supernatant fractions isolated and purified by the method of Schneider and Hogeboom(8) were added to 5 volumes of acetone:ethanol (1:1 v/v). After heating the preparation at 40° for one hour, precipitated protein was removed by filtration. Aliquots of the filtrate were used for isolation of fatty acids. Fatty acid preparations were methylated by a modification of the method of Stoffel *et al.*(9). Analyses of the mixtures of fatty acid esters were carried out using gas-liquid chromatography. A 4-foot column employing ethylene glycol adipate polyester (EGA) as the stationary phase and an argon ionization detector (W. G. Pye and Co., Cambridge, England) was used.

Results. Mitochondrial swelling. Results of the studies of swelling rates are illustrated in Fig. 1. In contrast to the low initial swell-

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‡ Abbreviations: EFA, essential fatty acids; EDTA, ethylene-diaminetetraacetate; Tris, tris(hydroxymethyl) aminomethane; ATP, adenosine triphosphate.

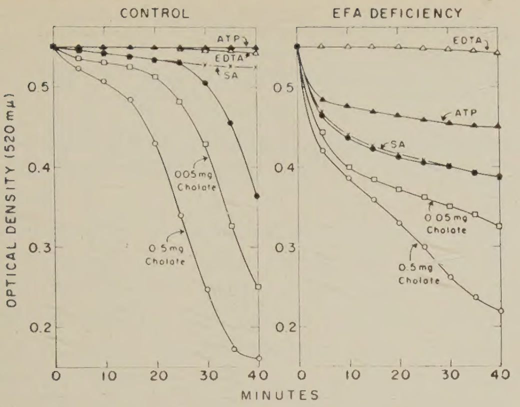

FIG. 1. Rates of swelling of liver mitochondria from rats fed a diet containing corn oil (5% of diet) and a diet deficient in essential fatty acids. Measurements were made at 520 mμ; temp., 23°-24°. The following additions were made to 0.30 M sucrose–0.02 M Tris, pH 7.4: ●——● no addition; ▲——▲ 0.003 M ATP; △——△ 0.001 M EDTA; ×——× 30 mg serum albumin per 3 ml media; □——□ 0.05 mg cholate per 3 ml media; ○——○ 0.5 mg cholate per 3 ml media.

ing rates of control mitochondria, markedly higher swelling rates during the first part of the observation period were seen for EFA deficient mitochondria. Relatively slower swelling rates of control and EFA deficient liver mitochondria were observed in 0.88 M sucrose than were seen using 0.30 M sucrose medium.

ATP prevented swelling of control mitochondria in 0.30 M sucrose medium, but not that of EFA deficient mitochondria. On the other hand, ATP appeared partially to inhibit swelling of control and EFA deficient mitochondria in a KCl media (results not indicated on figure). The different effects of ATP on mitochondrial swelling of EFA deficient mitochondria in sucrose and KCl media are reminiscent of the observations of Lehninger *et al.*(10) on thyroxine-treated mitochondria. EDTA, which was added to test the action of a metal chelating agent in the sucrose medium, showed a protective action against swelling of both groups of mitochondria. Thus, it appears that the mechanisms of action of ATP and EDTA as inhibitors of mitochondrial swelling are different. A similar conclusion was reached by Lehninger *et al.* (8) from studies of thyroxine-induced swelling.

Serum albumin (human, Cohn Fraction V) was added to the sucrose medium, to study the effect of a high molecular weight substance. Serum albumin prevented swelling of the control mitochondria during the latter part of observation period (Fig. 1); however, it did not prevent swelling of EFA deficient mitochondria. This observation suggested that permeability of the membrane of the liver mitochondrion was altered by feeding the fat-deficient diet. Serum albumin has been shown to act as a fatty acid binding agent; therefore, a possible explanation of the above phenomenon is that lack of EFA might influence permeability of the mitochondrial membrane by altering the binding sites for the albumin added to the test media.

Earlier studies have shown that certain detergents cause mitochondrial swelling(11,12). Potassium cholate, presumably a naturally-occurring anionic detergent in liver, accentuated the swelling pattern of mitochondria isolated from livers of each group of rats in this study. "Pluronic F68" (polyethylene propylene glycol), which is a non-ionic detergent, was also tested. The results were similar to those seen with cholate in that the shape of the swelling curves of the mitochondria from both groups of rats were merely accentuated.

The effects of various respiratory inhibitors, in presence of β-hydroxy-butyrate as a chain reductant, on the swelling of mitochondria from livers of the 2 groups of rats were also evaluated. Lehninger(13) has shown that cyanide, amytal and antimycin A inhibit mitochondrial swelling. All 3 agents were effective in preventing swelling of control mitochondria in these experiments and were only slightly less effective as inhibitors of swelling of EFA deficient mitochondria. All of the above phenomena were observed consistently, being characteristic of mitochondria isolated from 12 control and 12 EFA deficient rats.

Fatty acid analyses of mitochondria, microsomes and supernatant fractions. The distribution of fatty acids from total lipid extracts of mitochondria, microsomes and supernatant fractions isolated from livers of control rats and from rats fed diets deficient in essential fatty acids is indicated in Table I. Values are the mean figures for 2 animals from each group. The mitochondrial lipid from liv-

TABLE I. A Comparison of Effect of Feeding a Fat-Free and a Corn Oil Containing Diet to Rats on Fatty Acid Composition of Lipids of Mitochondria, Microsomes and the Supernatant Fraction Isolated from Liver.

Fatty acid	Mitochondria		Microsomes		Supernatant	
Chain length: unsat.	Control	EFA deficient	Control	EFA deficient	Control	EFA deficient
14:0	1.8*	.6	.3	.1	.5	.6
15:0	.0	.0	.1	.0	.4	.2
16:0	14.8	15.5	20.2	17.9	23.2	24.1
16:1	3.4	8.2	2.4	6.6	3.7	10.4
17:0	.0	.0	.3	.0	.0	.4
18:0	19.1	19.6	23.1	19.6	16.9	14.3
18:1	10.6	24.0	13.1	23.1	19.0	29.6
18:2	20.3	1.6	13.9	2.7	17.8	1.1
20:3(?)	.0	21.2	.0	22.2	.0	9.4
20:4	30.0	8.9	25.7	3.7	18.5	4.8

* Values expressed as a percentage of total fatty acids in the fraction.

ers of rats fed control (corn oil containing) diets had a higher percentage of fatty acids as linoleic and arachidonic acids than did either microsomal or supernatant fractions. These 2 acids made up 50% of the fatty acids of the control mitochondria. The feeding of diets essentially devoid of fat resulted in very low levels of linoleic and arachidonic acids in all 3 cellular subfractions. In agreement with the rather consistent observations of various workers in studies of essential fatty acid deficiency, the differences in total unsaturation of lipids from the 2 groups of rats were partially offset by the appearance of higher concentrations of endogenously derived monounsaturated fatty acids (*i.e.,* oleic and palmitoleic acids) in fractions from the rats fed fat-free diets. There were, furthermore, 2 twenty carbon acids in the chromatographs of methyl esters of fatty acids isolated from the liver cellular subfractions of rats deficient in essential fatty acids. The smaller of the 2 peaks had a retention time which was consistent with a 20:4 acid. The larger of the 2 peaks had a shorter retention time which was consistent with that of a 20:2 acid(14) but was probably 5, 8, 11-eicosatrienoic acid. After hydrogenation of lipid samples from the EFA deficient group, a single peak equivalent in size to the sum of the eicosatrienoic (20:3) and eicosatrienoic (20:4) peaks before hydrogenation appeared at the arachidic (20:0) acid locus.

Discussion. These studies indicate that *in vitro* patterns of swelling of normal mitochondria and mitochondria from rats fed diets deficient in essential fatty acids were different when a variety of suspending media and supplements were used. Fatty acid composition of lipids from control and deficient mitochondria were also strikingly different. The presence of high concentrations of linoleic and arachidonic acids in control mitochondria and of 5, 8, 11-eicosatrienoic in deficient mitochondria is largely consistent with the findings of Klein and Johnson(15) using the alkaline isomerization technic. Twenty-one per cent of the fatty acids in lipids of mitochondria from the deficient group appeared to be an eicosadienoic or eicosatrienoic acid of unknown specific structure.

It seems possible that differences in swelling of mitochondria from the 2 groups of rats may be related to differences in concentrations of the fatty acids in the lipids present in the mitochondrial membrane. The finding that anaerobiasis protects mitochondria against ageing(16) suggests that the protective effect of EFA may be related to their role as easily oxidizable fatty acids. The inhibitory effect of serum albumin, which has a strong affinity for free fatty acids, on swelling of normal, but not deficient, mitochondria could also be interpreted as resulting from the binding of albumin to sites on the mitochondrial membrane whose albumin binding capacities are influenced by their fatty acid composition.

Klein and Johnson(1) have shown that liver mitochondria from rats fed diets deficient in essential fatty acids uncouple oxidation from phosphorylation. Results of the present study demonstrated that ATP added to 0.30 M sucrose media inhibits swelling of mitochondria from the control but not the swelling of mitochondria from the deficient group. On the other hand, EDTA inhibited swelling of liver mitochondria from both groups of rats. These data would indicate that ATP influences mitochondrial swelling in a manner related to its role as a phosphate donor.

Summary. Comparison of rates of *in vitro* swelling of liver mitochondria from control rats and from rats fed diets deficient in essential fatty acids were studied under a variety of conditions. Higher swelling rates of EFA deficient mitochondria were observed using 0.30 M sucrose medium. Addition of ATP or serum albumin inhibited swelling of normal but not that of EFA deficient mitochondria. Respiratory inhibitors prevented swelling of both groups of mitochondria. Cholate accentuated the swelling pattern of mitochondria from both groups of rats. The fatty acid composition of lipid from mitochondrial, microsomal and supernatant fractions from both groups of livers were also presented.

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Linoleic Acid as Causative Agent of Encephalomalacia in Chickens Fed Oxidized Fats.[*] (25626)

L. J. MACHLIN AND R. S. GORDON (Introduced by F. J. Stare)
Lab. of Biochemistry and Nutrition, Monsanto Chemical Co., St. Louis, Mo.

Chickens fed certain partially oxidized fats manifest an edemic condition known as "toxic fat" disease(1,2,3). Our original purpose was to determine whether the feeding of heated cottonseed oil will result in this disease. It was found that ingestion of heated cottonseed oil does not cause "toxic fat" disease but does result in a high incidence of encephalomalacia even with dietary levels of Vit. E usually sufficient to prevent this disease. High levels of Vit. E or a supplement of the antioxidant Santoquin[†] (herein after referred to by generic term ethoxyquin) completely prevented encephalomalacia. Evidence is presented which demonstrates that increased requirement for Vit. E was caused by presence of linoleic acid in the diet. This is in agreement with other reports(4,5,6). Fats high in linolenic acid but

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[*] Preliminary report presented in *Fed. Proc.,* 1959, v18, 535.

[†] Registered trademark of Monsanto Chemical Co., for 1,2-dihydro-6-ethoxy, 2,2,4-trimethyl quincline.

TABLE I. Experimental Diets.

Ingredient	ES-45	ES-35
	%	
Isolated soybean protein[*]	45.	35.0
Fats	20.	4 to 16
Salts A[†]	6.	6.0
Microcel E[‡]	2.	
Glycine	1.4	1.0
Choline chloride mixture (25%)	.8	.8
Methionine hydroxy analog (MHA)[§]	.7	.7
Vit. mixture (triturated on glucose)[‖]	.5	.5
Vit. A conc. (10,000 I.U./g)[¶]	.1	.10
" D_3 " (7,500 ")	.03	.03
Aureomycin conc. (10 mg/lb)	.1	.10
Procaine, penicillin (50%)	.004	.004
NF 180[**]	.05	.05
Glucose (cerelose)	to 100	to 100

[*] Assay Protein C-1, Archer-Daniels-Midland Co., Cincinnati, O.

[†] Contains in g/60 g the following compounds: $CaCO_3$, 15; K_2HPO_4, 9; Na_2HPO_4, 7.3; $Ca_3(PO_4)_2$, 14; NaCl, 8.8; $MgSO_4 \cdot 7H_2O$, 5; ferric citrate, 0.5; $MnSO_4 \cdot 4H_2O$, 0.42; KI, 0.04; $ZnCO_3$, 0.02; $CuSO_4 \cdot 5H_2O$, 0.02.

[‡] Johns-Manville, N.Y.

[§] Registered trademark of Monsanto Chemical Co. for calcium *dl*-2-hydroxy, 4-methylthiobutyrate.

[‖] This supplies in mg/kg of finished diet: vit. B_{12}, 0.03; biotin, 0.3; menadione, 1; pyridoxine HCl, 8.; folic acid, 4.; riboflavin, 16.; calcium pantothenate, 20.; thiamine HCl, 24.; nicotinic acid, 100. This mixture and salts "A" can be obtained from Nutritional Biochemicals Corp., Cleveland, O.

[¶] A stabilized preparation prepared by Dawes Lab., Chicago, Ill.

[**] Registered trademark of Hess and Clark for furazolidine feed preparation.

low in linoleic acid had no effect on Vit. E requirement.

Methods. Cockerels from a Vantress-Arbor Acre cross were reared in electrically heated, wire-floored cages maintained in air-conditioned rooms provided with continuous illumination. Diets were fed *ad lib.* All birds were examined daily and those showing definite signs of encephalomalacia (ataxia, "cycling" motion of legs, head retraction, etc.) were removed. The brains of some affected birds were examined histologically. In almost all cases cerebellar lesions typical of those previously reported for encephalomalacia(7) were observed. In the first 2 experiments the chicks were fed a corn-soybean diet containing 7.7 I.U. Vit. E[‡]/kg for 7 to 10 days. They were then fed the experimental rations containing 20% heated cottonseed oil. The basal diet (S-45, Table I) contained no source of Vit. E. In all other studies a purified diet containing 4% "stripped" lard as the only source of Vit. E (less than .066 I.U. E/kg diet) was fed for 7 to 10 days. The birds were then fed experimental diets for 11 to 22 days. All fats were stabilized after heating with 0.1% Tenox VI.[§] Determination of peroxide level, as the TBA (thiobarbituric acid) value(8,9), of dietary fat at beginning and end of experiments demonstrated that no oxidative rancidity had occurred during this period. In addition to dietary source of Vit. A (10,000 I.U./kg) an emulsion of Vit. A acetate was added to drinking water as suggested by Bieri *et al.*(10). The livers of chickens receiving experimental diets, including those showing symptoms of encephalomalacia, contained 63 to 194 I.U. Vit. A/g when analyzed at end of experimental period. It is therefore believed that the studies were not complicated by Vit. A deficiency.

TABLE II. Effect of Feeding Heated, Aerated Cottonseed Oil on Incidence of Encephalomalacia.[*]

Fat source *Exp. 1*		Incidence of encephalomalacia or death[†]
Cottonseed oil (Wesson oil)		0/4
Idem	heated[‡] 95°C, 13 days	14/14
"	" 170-180°C, 4 days	14/14

[*] All birds were fed corn-soybean meal diet containing approximately 7.7 I.U. vit. E/kg for 10 days. They were then fed experimental diets for 22 days. Diet S-45 with addition of 8.8 I.U. vit. E/kg was used as the basal.

[†] Numerator is No. of chickens which either died or showed definite symptoms of encephalomalacia after start of experiment. Denominator is No. of chickens started on each treatment.

[‡] 2 to 4 kg were heated in stainless steel beakers with air bubbling through pot at 500-700 ml/min.

Results. In Exp. 1 (Table II) all birds died or showed definite signs of encephalomalacia when fed heated aerated, refined cottonseed oil. In agreement with Naber(2) no birds showed symptoms of "toxic fat" disease. Since encephalomalacia can be prevented by Vit. E or certain antioxidants(8,11), the next studies were designed to determine levels of Vit. E and/or antioxidant required to prevent the disease when 20% heated cottonseed oil was fed. The requirement for ethoxyquin was between 0.0125% and 0.02% of the diet, (Table III). The antioxidant BHT at a level of 0.05% had no effect on the disease. The requirement for Vit. E was more than 44 but less than 88 I.U./kg. This is much higher than any previously reported requirement.

[‡] As d α tocopherol acetate.

[§] This product contains 10% BHT and 10% BHA. Available from Eastman Chemical Products, Inc., Kingsport, Tenn.

TABLE III. Effect of Vit. E and Ethoxyquin on Encephalomalacia Induced by Feeding of Heated Cottonseed Oil.

Fat source	Vit. E (I.U./kg)	Ethoxyquin (%)	Incidence of encephalomalacia
_Exp. 2_*			
Cottonseed oil	22		0/11
Heated cottonseed oil†			11/11
Idem	22		7/11
"	220		0/11
"		.05	"
"	22	.05	"
" + .05% BHT‡			10/11
Exp. 3a§			
Cottonseed oil			0/10
Heated cottonseed oil†			9/10
Idem	44		2/10
"	88		0/10
"	176		"
"		.0125	1/10
"		.0250	0/10

* Chicks fed a corn-soybean diet containing 7.7 I.U. vit. E/kg for 7 days, then fed experimental rations for 15 days.

† Heated and aerated at 170-180°C for 4 days.

‡ 2,6-ditertiary-butyl-p-cresol.

§ All birds fed vit. E deficient diet for 8 days, then fed experimental diets for 15 days. 0.1 ppm of selenium (as $Na_2Se_2O_3 \cdot H_2O$) was added to basal diet. After heating, 0.1% of Tenox 6 (an antioxidant containing 10% BHT and 10% BHA) was added to the fat.

Studies were then conducted to determine whether feeding of other heated fats would also result in a high incidence of encephalomalacia. In all cases analysis of heated fats for Vit. E(12) demonstrated that essentially all the Vit. E had been destroyed. The results summarized in Table IV show that the encephalomalacia-inducing ability of heated cottonseed oil was not unique and that in general the incidence of encephalomalacia was correlated with linoleic acid content of fats. Linseed oil which is much more unsaturated than cottonseed oil resulted in a lower incidence of encephalomalacia.

TABLE IV. Correlation of Linoleic Acid Content of Various Oxidized Fats with Encephalomalacia.

Dietary fat	Iodine No.	Linoleic acid (%)	Encephalomalacia (index)*
Safflower oil	140	72.4	152 (2)
Cottonseed "	114	54.8	100 (3)
Corn "	127	55.7	57 (2)
Peanut "	101	29.0	14 (1)
Linseed "	180	18.0	16 (3)
Olive "	84	7.0	0 (1)
Tallow	40	2.2	0 (1)
Cod liver oil	165	2.3	0 (1)

* Index =

$$\frac{\%\text{ encephalomalacia in test fat} \times 100}{\%\text{ encephalomalacia in cottonseed oil group}}.$$

No. in parenthesis indicates No. of experiments conducted.

Fatty acids of cottonseed oil were then fractionated after conversion of the triglycerides to the methyl esters by interesterification with methanol using sodium methylate as catalyst. The methyl esters were fractionally distilled using a 2 foot Vigreaux column (2-3 theoretical plates) operating at pressure of approximately 0.1 mm mercury. The fractions were analyzed by gas chromatography and fed to 1 week old chickens. The results are given in Table V. They show that encephalomalacia results from feeding of distilled methyl esters and that incidence of encephalomalacia was correlated with linoleic acid content of the fraction fed. As fractional distillation procedure did not separate oleic and linoleic acid to any appreciable extent, in the next study these fatty acids were fed separately. These results are given in Table VI

TABLE V. Fractionation of Cottonseed Oil.*

Fraction #	Linoleic	Oleic	Palmitic	Stearic	Incidence of encephalomalacia†
	%				
1	32.4	5.2	53.9	.7	0/3
2	36.4	11.3	49.0	1.3	1/3
3	64.1	20.5	12.3	2.4	1/3
4	72.5	23.4	.3	3.3	3/3
Residue	67.3	25.4		5.3	2/3

* Distilled at 0.1 mm mercury pressure with 2 ft Vigreaux column. All fractions fed at 8% of diet (ES-35) for 7 days, after 7 days on vit. E-free diet (ES-35 containing 4% stripped lard).

† Numerator is No. of birds showing definite symptoms of encephalomalacia. Denominator is No. of birds/treatment.

TABLE VI. Incidence of Encephalomalacia in Chickens Fed Purified Fatty Acids.

Dietary fat	Incidence of encephalomalacia*
None (Diet ES-35)	0/10
5% linoleic acid†	6/6
10% oleic " ‡	0/8
20% " "	0/4

* Numerator is No. of birds showing definite symptoms of encephalomalacia. Denominator is No. of birds/treatment.

† Obtained from Hormel Fn., Austin, Minn. Lot 15-M. Iodine number is 172.5 (theoretical 172.4). A single peak observed with gas chromatography.

‡ Obtained from Fisher Chemical Co. Catalog No. A-222. Contains less than 5% polyunsaturated fatty acids.

and clearly demonstrate that feeding of linoleic but not oleic acid resulted in encephalomalacia.

Feeding of heated cod liver oil also did not result in encephalomalacia. This fat contains arachidonic and clupanodonic acid. These highly unsaturated compounds could have been destroyed during the heating process. To avoid such destruction a sample of cod liver oil was saponified(13) in the presence of 0.1% ethoxyquin. The soap solution was extracted for 24 hours with ether(13). This removed all Vit. E. The soaps were then converted to fatty acids by addition of acid and fed. At the end of 20 days, 10 out of 16 birds fed 12% cod liver oil fatty acids had encephalomalacia.

Discussion. These studies demonstrate that addition of linoleic acid to diets low in Vit. E and other biologically active antioxidants results in encephalomalacia. Fats high in linolenic acid were less effective than fats high in linoleic acid. Our observations as those of Century and Horwitt(14), confirm the work of Dam *et al.*(4), who reported that feeding of linoleic acid, but not linolenic acid, results in encephalomalacia. However, our work is not complicated by a selenium deficiency or by possible oxidation of fatty acids in the diet and gut.

Although not definitely proven, arachidonic acid also appears to exaggerate Vit. E deficiencies. Dam(15) showed that feeding of fatty acid fractions known to be high in arachidonic acid promoted encephalomalacia. In the present study, cod liver oil subjected to procedures which removed Vit. E but which did not destroy arachidonate caused encephalomalacia. In addition Moore and his co-workers(16) have shown that cod liver oil acts as a Vit. E antagonist in the rat. Century and Horwitt(16), however, found that cod liver oil did not promote encephalomalacia. Since no precautions were taken in their experiments to prevent oxidation of fatty acids after addition to the diet, destruction of some or all of the arachidonic acid before absorption could have accounted for their results.

The effect of linoleic and presumably arachidonic acid in promoting Vit. E deficiency symptoms could be the result of: (1) destruction of Vit. E in the diet and in the gut during oxidation of these fatty acids, (2) increased destruction of Vit. E in tissues due to presence of high levels of these fatty acids and their subsequent autoxidation, (3) disruption of some cell structure and/or metabolic process as a result of autoxidation of linoleic and/or arachidonic acid in the tissue.

Blaxter *et al.*(17) reported that dystrophy of calves fed cod liver oil is prevented by methylene blue without a concomitant increase in tocopherol content of the tissues. Jensen *et al.*(18) found that eggs from turkeys fed fish oil failed to hatch unless DPPD (N,N′ diphenyl-p-phenylenediamine) was added to the diet. Addition of DPPD did not cause increased tocopherol deposition in the egg yolk. Draper and Csallany(19) demonstrated quite clearly that the effect of DPPD in curing muscular dystrophy in the rabbit was not due to conservation of Vit. E in the tissues or in the diet. Most of our diets were essentially devoid of Vit. E. Moreover, destruction of Vit. E in the diet (and presumably in the gut) was prevented by addition of an antioxidant. These observations indicate that destruction of Vit. E in the diet, gut or tissue is not a likely explanation for our observations. We think a more reasonable hypothesis is that addition of linoleic and/or arachidonic acid to the diet increases the content of these fatty acids in the tissue. Their subsequent autoxidation following depletion of antioxidants from tissues results in disruption of a critical cell structure which is the beginning of a sequence of events which results in encephalomalacia.

The observation that linolenic acid does not promote encephalomalacia would be explained if linolenic acid was not a part of the critical lipid structures involved, or if deposition of this particular fatty acid in the tissues was not increased significantly by its addition to the diet.

Summary. Feeding of heated and aerated unsaturated fats to chickens fed Vit. E low diets resulted in high incidence of encephalomalacia. Incidence of encephalomalacia was correlated with linoleic acid, but not the linolenic acid content of fats fed. Feeding of highly purified linoleic acid resulted in 100% incidence of encephalomalacia. When 20% heated cottonseed oil was the fat source between 44 and 88 I.U./kg of Vit. E or between .0125% and 0.025% of the antioxidant, ethoxyquin was required for prevention of the disease.

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Chelation and Iron Metabolism I. Relative Iron Binding of Chelating Agents and Siderophilin in Serum.* (25627)

MARTIN RUBIN, J. HOULIHAN† AND JOSEPH V. PRINCIOTTO
Depts. of Biochemistry and of Physiology and Biophysics, Georgetown University Schools of Medicine and Dentistry, Washington, D.C.

Iron absorption, transport, storage and function are mediated in the organism by the nature of the iron combination. It may be anticipated that knowledge of the factors governing iron complex formation and interaction can lead to new approaches to problems of iron therapy(1,2,3,4). The present study concerns the relative iron-binding strengths of the plasma iron-binding protein siderophilin and various synthetic iron-binding chelating agents in rabbit serum.

Materials and methods. The synthetic chelate compounds, beta hydroxyethyliminodiacetic acid,‡ nitrilotriacetic acid,‡ dibetahydroxyethylglycine,‡ 1,2-propylenediaminetetraacetic acid,§ betahydroxyethylethylenediaminetriacetic acid§ (HOEDTA), ethylenediaminetetraacetic acid|| (EDTA), and trans-1,

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2-diaminocyclohexanetetraacetic acid,[‖] were provided as the pure crystalline products. Stock solutions of the compounds were prepared at a concentration equivalent to an iron-binding strength of 500 γ/ml in appropriate volumes of iron-free water. The solution pH was adjusted to 7.0-8.0 by addition of dilute iron-free sodium hydroxide. For use the stock solutions were diluted to an iron-binding equivalent of 50 γ/ml. Standard stock solutions of iron chelates were prepared by mixing equal volumes of the stock standard chelate solutions and a standard solution of 500 γ/ml of iron prepared by solution of iron wire in dilute hydrochloric acid. The stock standard iron chelate solutions were converted to working solutions at an iron concentration of 50 γ/ml by dilution with iron-free water and pH adjustment to 7.0 immediately prior to use. Serum was prepared from blood obtained by cardiac puncture in the 24-hour fasted rabbit using a siliconized syringe and needle. Determination of the unsaturated iron-binding capacity (UIBC) of an aliquot of each serum sample was carried out by the Ventura modification(5) of the Rath and Finch procedure(6). Division of total optical density change in determination of UIBC by the total gamma of iron added provided a measure for each serum sample of the optical density change for the iron siderophilin formed for a gamma of added iron. This baseline value served as a convenient standard to measure either removal of iron from iron siderophilin or efficiency of formation of the iron protein complex under experimental conditions. The iron chelates utilized in the study showed no absorption in serum at maximum concentrations and wave lengths used in the analyses.

Results. A. *Removal of iron from iron-siderophilin by chelating compounds in serum.* Serum brought to 100% of its iron-binding capacity by addition of increments of standard iron solution was treated with each solution of the enumerated chelate compounds to an equivalent iron-binding of 10-fold the total serum iron content. Other than the minor optical density change resulting from dilution there was no change in absorbancy of the iron protein complex. There was no change in optical density during a 24-hour storage period.

B. *Removal of iron from iron-chelates by siderophilin in serum.* The total amount of iron calculated to be required to saturate UIBC of the serum sample was added in increments in the form of standard iron chelate solutions. Transfer of iron from chelate to iron-binding protein was followed by the increase in optical density due to the iron protein complex. Iron added as the betahydroxyethyliminodiacetic, dibetahydroxyethylglycine and nitrilotriacetic acid chelates was immediately and completely transferred to the protein. Iron added in the form of the other chelates of this study did not increase absorbancy due to the iron protein in an observation period of 12 hours. Typical data for the observed optical density changes are given in Table I.

TABLE I. Comparative Optical Density Data Showing Typical Examples of Results Obtained in Rabbit Serum.

Gamma Fe added		Optical density at 520 mu		
		Cell 1	Cell 2	Cell 3
1. Fe⁺⁺	.0	.053	.058	.056
	1.0	.065	.070	.068
	2.0	.077	.082	.080
	3.0	.081	.086	.084
2. Fe-β-hydroxyethylimino-diacetic acid				
	.0	.062	.058	.059
	.9	.072	.067	.069
	1.8	.083	.077	.080
	2.7	.084	.081	.082
	3.6	.084	.081	.082
	12 hr later	.085	.082	.083
3. Fe-ethylenediamine-tetraacetic acid				
	.0	.061	.061	.061
	.9	.062	.061	.061
	1.8	.061	.060	.060
	2.7	.060	.059	.059
	3.6	.059	.058	.058
	12 hr later	.059	.058	.058

C. *Distribution of added iron between siderophilin and synthetic chelates in serum.* Iron was added in one gamma increments to serum samples containing the chelating compounds in quantities equal to twice the total iron-binding capacity of the serum as measured by the UIBC of an aliquot. Under these conditions only HOEDTA demonstrated ability in a 12-hour observation period to bind

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[‖] Provided by Mr. H. Zussman, Geigy Chemical Co.

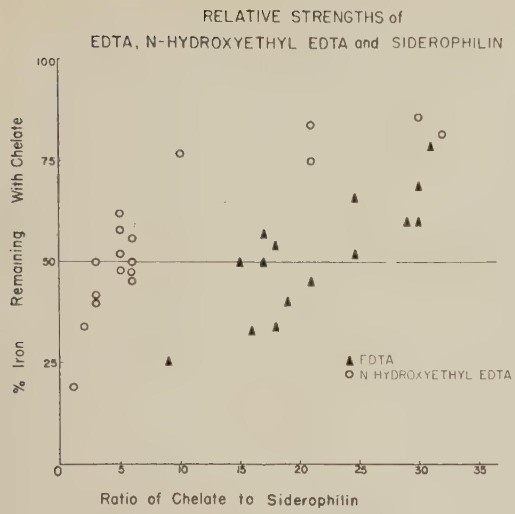

FIG. 1. Distribution of iron added to rabbit serum containing various concentrations of EDTA or N-HOEDTA.

iron in competition with the iron-binding protein of the serum.

The competition between chelate compound and iron-binding protein for iron added to serum was examined for a wide range of chelate/protein equivalent iron-binding ratios for the compounds EDTA and HOEDTA. The results of these measurements are presented in Fig. 1 in terms of percentage of added iron bound by the chelate. It is evident that at the equivalent iron-binding ratio of 3-5/1 for HOEDTA and 15-25/1 for EDTA, distribution of iron added to serum is equal between synthetic chelates and iron-binding protein.

Discussion. Results of this study illustrate problems encountered in application of synthetic metal-binding agents to biological systems. They also provide background information for solution of some of the problems of iron metabolism.

The calculated "stability constant" values provide a useful measure and relative guide of extent of metal-chelate interaction(7). Several experimental conditions are implicit in calculation of these values. Equilibrium reaction conditions, complete ionization of the organic ligand and absence of competing anions or ligands are requisite baseline factors in these measurements. In biologic systems, at the essentially neutral pH range, the kinetics of the reactions of metabolism, distribution and excretion of the metal or the chelating agent may take precedence over and mitigate against attainment of metal-chelate equilibrium.

The present study, using rabbit serum, has equalized the action on all the compounds of the variables of pH, the competitive influence of naturally occurring ligands and anions, and the kinetics of these related reactions on the iron-binding reaction under investigation. In consequence, the effective rather than the theoretical iron-binding ability of the compounds has been determined compared to the iron-binding protein of the blood.

None of the available compounds was able to remove iron from iron-siderophilin in serum. This first indication that synthetic compounds were less effective iron-binding agents than iron-binding protein was not fully substantiated by the next phase of this investigation. While the iron chelates of the ammonia acetic acid series delivered their iron to the protein promptly as might be expected from the first phase of the study, the iron of the iron chelates in the ethylenediamine series was retained by the synthetic compounds. Thus an equilibrium could not be attained in this series in the reaction

Fe siderophilin + chelate $\leftrightarrows$ Fe chelate + siderophilin by approaching from either side of the equation. This result is not entirely unexpected. While the reaction of combination of metal ion and binding agent is usually quite rapid, the exchange reaction of metal ion from one chelate to another can be slow. This is especially the case for exchange of metal between binding compounds of the same relative order of binding strength(8). In an attempt to obviate this kinetic difficulty the study of distribution of added metal ion between competing ligands was performed to gain some insight into the effective iron-binding ability of EDTA and HOEDTA *vis a vis* siderophilin in serum. The results (Section C) indicate that HOEDTA is a somewhat more effective iron-binding agent than EDTA under the conditions present in serum. This finding is in accord with the physical chemical evidence which supports the superiority of the iron-binding ability of HOEDTA in an alkaline medium(9).

Studies of metabolism of parenterally administered EDTA in animals and humans(10, 11) have demonstrated a biological half-life of approximately 30 minutes. When coupled with the present work this would suggest that the synthetic chelates would be limited in iron combination *in vivo* to those areas of the body where they would be in direct competition with the iron-binding protein for "free" ferric or ferrous ions. The tissue iron storage areas are considered to be in this category(12). Here the synthetic chelate could bind iron in competition with the unsaturated iron-binding protein to an extent governed by competitive iron-binding ability, effective compound concentration, size of iron storage, and metabolic fate of the iron chelate.

Summary and conclusions. 1. The iron-binding ability of a series of synthetic chelating agents has been compared to that of siderophilin in rabbit serum. 2. The chelate compounds are unable to remove iron from siderophilin in rabbit serum. 3. Under the same conditions siderophilin is unable to remove iron from the more tightly bound iron chelates. 4. EDTA and HOEDTA, at iron-binding ratios of 20-25/1 and 3-5/1 respectively to that of the protein, compete on equal basis for iron added to rabbit serum.

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Connective Tissue II. Effect of Age and Sex upon Lipid Composition of Tissues of Rat.* (25628)

KUNG-YING TANG KAO, JOY H. SCHWARTZ, CARLETON R. TREADWELL AND THOMAS H. MCGAVACK

Geriatrics Research Labs., Vet. Admin. Center, Martinsburg, W. Va., Depts. of Biochemistry and Medicine, George Washington University School of Medicine, Washington, D.C.

Further to correlate the behavior of lipids and protein in connective tissue, the present study was undertaken to determine the effect of age and sex upon: (1) Free cholesterol, (2) Total cholesterol, (3) Phospholipids, and (4) Triglycerides in the connective tissue of various structures of the rat.

Procedures. Male and female rats of the Wistar strain, from 4 weeks to 2½ years old were used in this study. One-tenth to 0.5 g of tail tendon, aorta, skin and uterus, respectively, were cut into small pieces and heated to boiling with 20 ml portions of 1:1 acetone-alcohol, 3 times, and with 3:1 ether-alcohol, twice. After each extraction, the extracting tubes were centrifuged at 2500 rpm for 5 minutes. In each instance, the supernatant was filtered. The combined extracts were evaporated on a steam bath to dryness and made up to a known volume with a 2:1 methyl alcohol-chloroform mixture. Aliquots were taken for determination of free and total cholesterol, phospholipids and total lipids. Free cholesterol was precipitated as digitonide and determined colorimetrically by the method of

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TABLE I. Effect of Age and Sex upon Lipid Composition of Skin of Rat (mg/g of Tissue).

	Age (wk)	Cholesterol		Phospholipids	Triglycerides
		Free	Total		
♂	4-5 (30) A	1.8 ± .5 BCDE <.001	3.3 ± .6	7.6 ± 1.1 BCDE <.001	45.2 ± 17.8 BC <.001
	7-11 (24) B	1.3 ± .2 CDE <.001	3.1 ± .6	5.7 ± 1.4 CDE <.001	98.0 ± 43.3 D <.05 E <.01
	17-26 (12) C	.9 ± .1 DE <.05	2.8 ± .7	*3.3 ± 1.6*	111.3 ± 51.0 E <.01
	30-34 (12) D	*.8 ± .1*	2.3 ± .5	3.5 ± 1.3	*69.0 ± 20.5*
	130 (10) E	.8 ± .1	*3.1 ±1.0*	3.0 ± .6	*52.5 ± 16.1*
♀	4-5 (30) A	2.0 ± .2 BCDE <.001	3.2 ± .4 C <.05 DE <.001	8.0 ± 1.6 DE <.001	55.0 ± 23.0 BCDE <.001
	7-11 (20) B	1.4 ± .3 CDE <.001	2.7 ± .5 C <.01 DE <.001	6.0 ± 1.1 DE <.001	127.0 ± 64.0
	17-26 (15) C	1.0 ± .1	2.2 ± .6	*6.2 ± 1.4* D <.001 E <.01	175.0 ± 42.0
	30-34 (16) D	*1.0 ± .1*	2.0 ± .5	3.4 ± .9	*179.0 ± 54.0*
	130 (9) E	.9 ± .1	*1.7 ± .1*	3.4 ± 1.4	*138.0 ± 41.0*

Figures in parentheses represent No. of animals and corresponding No. of determinations unless otherwise specified.

± indicates stand. dev. of mean.

P value relating to age differences, when within 5% level of confidence, is listed beneath results of actual determination and represents a comparison between that figure and that of age groups designated by letter or letters preceding the value.

Italicizing designates significant P values within 5% level of confidence relating to sex differences.

Sperry and Webb(1). The ester cholesterol was hydrolyzed and determined in the free state by the method just mentioned. Phospholipids were determined by the method of Fisk and Subbarow(2); total lipids by the Bragdon dichromate oxidation procedure(3); and triglycerides calculated by difference as described by Bragdon(3).

Results. The effects of age and sex upon composition of lipids in the skin, tendon and uterus of rats are recorded in Tables I to III, inclusive. In tendon no significant changes were observed beyond the 17 - 26 week period; therefore, data beyond this have been omitted from the tables. For the same reason uterine data beyond the 8th month have been omitted from Table III. The only significant changes observed in aortic lipids are concerned with esterified cholesterol. Therefore, no table has been constructed on the data in this structure. In the male, esterified cholesterol ranged from .11 ± .05 mg/g at 4 - 5 weeks to .22 ± .03 at 2½ years. For the female, corresponding figures were .01 ± .02 to .22 ± .09. In the male, most of this change appeared between 7 and 11 weeks, whereas in the female it was not observed until the 17th - 26th week. Initial figures for the other lipids in the aorta of the male rat were in mg/g of tissue: Free cholesterol 1.5 ± .5, total cholesterol 1.6 ± .5, phospholipids 9.5 ± 5.8 and triglycerides 34.8 ± 22.0. The female figures did not differ significantly from those quoted for the male. In making these determinations from the aorta, all specimens were pooled from 2 - 3 rats and from 2 - 9 determinations were made at each of the 5 age levels mentioned in Table I.

Variations in tissue lipids with age included:

a. In skin, a decrease in free cholesterol in both sexes, and also of total cholesterol in the

TABLE II. Effect of Age and Sex upon Lipid Composition of Tendon of Rat (mg/g of Tissue).

	Age (wk)	Cholesterol Free	Cholesterol Total	Phospholipids	Triglycerides
♂	4-5 (15)* A	2.5 ± 1.1 B <.02 CDE<.001	2.5 ± 1.1 B <.01 CD<.001	8.9 ± 3.3 B <.01 CD<.001	20.6 ± 13.7
	7-11 (6)* B	1.2 ± .5 D <.05	1.2 ± .4 D <.05	4.2 ± 2.8	13.2 ± 4.7
	17-26 (6) C	.7 ± .4	.8 ± .1	2.3 ± .7	8.6 ± 1.5
♀	4-5 (12)* A	1.9 ± .2 BCD <.001	2.1 ± .5 CD <.001	7.1 ± 1.6 BCD <.001	36.0 ± 25.2
	7-11 (7)* B	1.3 ± .4 D <.01	1.4 ± .4 D <.01	4.3 ± 1.1 CD <.001	21.3 ± 11.4
	17-26 (5) C	.7 ± .5	.8 ± .5	2.3 ± .7	11.2 ± 2.1

See legend, Table I.

* Each determination consists of a pooled sample from 2-3 rats.

TABLE III. Effect of Age upon Lipid Composition of Rat Uterus (mg/g of Tissue).

	Female			
Age (wk)	Cholesterol Free	Cholesterol Total	Phospholipids	Triglycerides
4-5 (8)* A	2.4 ± .3	2.7 ± .4	12.5 ± 2.9 D <.001	27.2 ±15.5
7-11 (7)* B	2.4 ± .8	2.8 ±1.0	12.0 ± 3.2 D <.001	15.1 ± 8.5
17-26 (5) C	2.2 ± .0	2.5 ± .2	9.9 ± 2.7	9.9 ± 2.6
30-34 (8) D	2.2 ± .4	2.4 ± .5	6.3 ± 1.4	15.3 ± 5.6

See legend, Tables I and II.

female; a decrease in phospholipids; and an increase in triglycerides in both sexes;

b. in tendon, an increase in ester cholesterol and a decrease in free and total cholesterol and in phospholipids in both sexes; and a decrease in triglycerides in the female;

c. in aorta, an increase in cholesterol esters; and

d. in uterus, a decrease in phospholipids. Sex differences were observed only in skin. Values for free cholesterol, phospholipids and triglycerides were higher in female than in male skin; the reverse was true for the value of cholesterol esters.

Comments. On analysis of whole rat carcass, Williams and his associates found changes between birth and 70 days of age similar to those recorded here(4). However, if determinations were limited to parenchymatous structures, they noted that cholesterol and phospholipids were increased(5). In the muscle of several species of animals, cholesterol has been observed to decrease with age (5,6). In bone marrow, several workers have found a decrease in lecithin with age(7,8,9). An increase with age in the lipids of renal epithelium has been reported(10).

Our findings of higher values for free cholesterol, phospholipids and triglycerides in the skin of female than in that of male rats agree well with those of Taylor and his associates(11). In the aortic tissues of the rat, the lack of a significant increase of cholesterol or neutral fat observed in these studies is in sharp contrast to corresponding data for chicken(12), horse(13) and man(14). Unlike skin and tendon, there is no decrease as a function of age in the cholesterol of the aorta.

Summary. 1. Changes as a function of age in values for free cholesterol, total choles-

terol, phospholipids and triglycerides in lipid extracts of rat tissues occurred between 4 weeks and 8 months of age, with no further changes, except in skin, at 2 years of age. 2. Sex differences in lipid composition occurred only in skin.

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Inhibition of Release of Intracellular Potassium by Non-Narcotic Analgesic Agents. (25629)

FRED B. BENJAMIN* (With technical assistance of V. Monteleone)
Colgate Palmolive Co., New Brunswick, N. J.

It has been shown that all types of pain-producing stimuli also release intracellular potassium (K) and that inhibition or reversal of K release is associated with decreased pain sensation(1). This new approach to pain might have some bearing on the action of analgesic agents. Therefore the present investigation is an attempt to determine whether non-narcotic analgesic agents inhibit release of intracellular K.

Two *methods* were used to produce a controlled release of intracellular K. The first one is distention of the intestine of guinea pigs. This procedure is well established as a painful stimulus(2,3). However K release under these conditions has not been shown previously. The other procedure used was exposure of washed erythrocytes to cold. In this case K release is a well established fact (4,5), but the relation to pain is purely by analogy. In the first procedure a guinea pig is killed by a knock on the head. The abdominal cavity is opened and 10 pieces of intestine, 2½ inches long, are cut. Intestinal chyme is gently pressed out. Each piece is washed in physiological saline and weighed (wet weight). One end of the strip is tied off with heavy nylon thread. The other end is tied to a glass rod. These rods have been prepared previously from glass tubing, external diameter 6.2 mm and internal diameter 5 mm. The rods are tapered and a small bulb is blown at the end to provide firm attachment for the strip (Fig. 1). The preparations are then transferred into 50 ml test tubes filled with 20 ml of K free Tyrode solution. Control experiments showed that there is a gradient of increasing K release down the intestinal tract (Table I). Therefore experimental preparations containing the analgesic agent are alternated with control tubes. The whole set of tubes is then transferred to an automatically stirred water bath kept at 37°C. After a control period of 30 minutes the intestine is distended with Tyrode solution (Fig. 1) until a steady fluid pressure of 2.5 cm is obtained. Samples are taken immediately after distention, one hour later and 2 hours

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* Present address: Republic Aviation Corp., Farmingdale, L.I., N.Y.

TABLE I. Gradient of K Release along Intestinal Tract. (5 pieces taken from successively lower levels.)

	½ hr control	S.E.	1 hr after distention	S.E.	2 hr after distention	S.E.
1	269	29	742	35	903	19
2	312	29	803	31	934	37
3	331	29	859	42	995	33
4	384	37	888	45	988	39
5	418	40	929	61	1,082	42
Mean	343	22	844	31	980	27
Mean without distention	339	18	603	26	689	17

Values indicate K concentration of bathing fluid (microequivalents/g dry wt of intestine) at given time and represent mean of 10 experiments with distention, and 4 experiments without distention.

later. For each test 2 ml of external fluid are withdrawn and replaced by 2 ml of K free Tyrode solution. In calculations adjustment is made for the resulting dilution. Each sample is diluted with an equal volume of 1,600 p.p.m. lithium chloride and analyzed for K in a Perkin-Elmer flame photometer using indirect technic. At the end of experiment the strips are removed from the glass rods, cut open and washed in saline, then dried by exposure to an infra-red lamp at a distance of 10 inches for 2 hours. Dry weight is determined and the tissue is macerated and extracted in cold water (20 ml). Extraction fluid is filtered and analyzed for K.

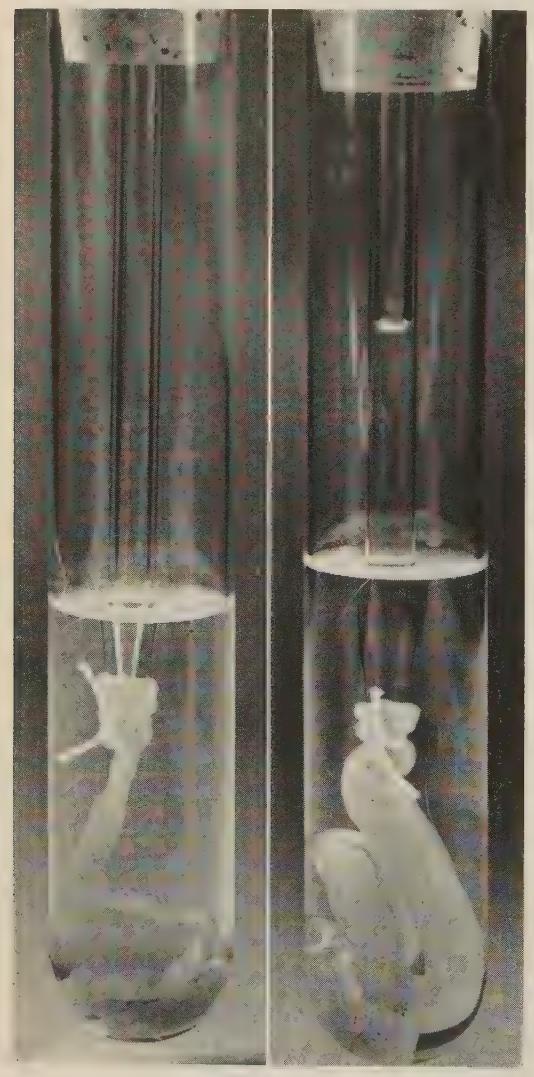

FIG. 1.

TABLE II. Effect of Drug Concentration on Inhibition of K Release.

Drug conc., mg/ml	No. exp.	1 hr after distention	S.E.	2 hr after distention	S.E.
.005	4	98.7	7.1	101.9	8.2
.01	4	79.9	6.4	87.3	5.9
.05	9	56.6	4.9	77.0	5.1
.5	4	87.0	8.0	92.1	7.7

Values for 3 drugs (phenacetin, acetanilid, aminopyrine) are combined and expressed as % of control.

Results. Table II gives the effect of various drug concentrations. In higher concentrations of drugs there was a slight change of pH which was corrected by adding $NaHCO_3$.

If we assume that the therapeutic dose in man is equally distributed throughout the extracellular fluid space, concentration would be about 0.015 mg/ml. This corresponds fairly well to the dose of maximum effectiveness in our experiments.

Tables III and IV give the results of 9 experiments analyzed according to wet weight

TABLE III. Effect of Analgesic Agents on K Release (Wet Wt).

	Control	Phen.	Control	Acet.	Control	Amin.
1 hr after distention	407	224 †	388	231 †	329	182 †
S.E.	74	44	60	29	44	67
% of control		55.		59.4		55.3
2 hr after distention	808	711 *	787	554 †	783	568 *
S.E.	102	49	61	70	85	73
% of control		88.		70.4		72.5

Values expressed as microequivalents/g wet wt, and are means of 9 experiments. S.E. refers to stand. error within the group, while significance indicated by asterisk and dagger is based on paired analysis.

* 95% probability. † 99% probability.

TABLE IV. Effect of Analgesic Agent on K Release (Dry Wt).

	Control	Phen.	Control	Acet.	Control	Amin.
1 hr after distention	3.63	2.23†	4.41	2.96†	3.80	2.68*
S.E.	.36	.33	.49	.31	.47	.63
% of control		61.4		67.1		70.5
2 hr after distention	7.60	6.40*	9.94	6.54†	8.58	7.06*
S.E.	.59	.56	.39	.56	.62	.76
% of control		84.2		65.8		82.3

Values expressed as milliequivalents/g dry wt and represent means of 9 experiments. S.E. refers to stand. error within the group, while significance indicated by asterisk and dagger is based on paired analysis.

* 95% probability. † 99% probability.

and dry weight respectively. Mean dry weight is 13.3% of mean wet weight.

Analysis of the intestinal wall showed that mean concentration for 4 controls was 2.60 meq/l and mean for 6 preparations with analgesic was 5.01 meq/l.

To determine effect of analgesics on K release from blood cells, washed human erythrocytes were suspended in physiological saline. The concentration was approximately 125 million cells/ml. Analgesic agents were used in a concentration of 0.05 mg/ml. Two tubes were used for each analgesic agent and a total of 4 controls. All tubes were exposed to a temperature of 7°C for 60 minutes. Then they were centrifuged at the same temperature and supernatant fluid was analyzed. Results in microequivalents/l showed:

Acetanilid 172	Phenacetin 231
Aminopyrine 273	Control 494

A problem arising from these data is the degree of specificity of inhibition of K release for analgesic agents. Table V shows that salicylic acid and cholesterol have no significant effect. Obviously only a greatly extended series of experiments can answer this point adequately.

TABLE V. Effect of Salicylic Acid and Cholesterol on K Release of Intestine.

Agent	Conc., mg/ml	No. exp.	1 hr after distention	2 hr after distention
Sal. acid	.25	9	99.2	98.0
Cholesterol	.25	6	96.1	97.8

All values expressed as % of control and none are significantly different from control.

Summary. In vitro distention of intestinal tract of guinea pigs causes release of intracellular K. This effect is decreased when bathing fluid contains non-narcotic analgesic agents (acetanilid, phenacetin, aminopyrine) in pharmacological concentrations. The release of K on exposing washed human erythrocytes to cold is similarly inhibited by these agents.

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Regeneration in Hypophysectomized Anuran Tadpoles.* (25630)

Margaret R. Wright and Mary Ellen Plumb (Introduced by L. S. Stone)
Department of Zoology, Vassar College, Poughkeepsie, N. Y.

The concept that normal regeneration in Amphibia depends in part upon the pituitary gland derives largely from experiments on the urodele limb(1-6). Lens regeneration, however, takes place in absence of the pituitary in *Triturus v. viridescens*(7-9) and retina regeneration(8) as well as regeneration of iris (9) occur in hypophysectomized newts. To account for the contrasting effects of hypophysectomy on limb regeneration and retina-iris-lens regeneration it may be useful to consider the effects of hypophysectomy on regeneration of other parts. It is well known that the tail-tip of the anuran tadpole regenerates, and that, as in the limb, the stimulus is amputation. There is much more similarity in mode of regeneration between limb and tail than between limb and lens. But, in contrast to the limb response in Triturus, the anuran tail-tip regenerates in hypophysectomized animals. This information was obtained incidentally in experiments on regeneration of denervated lateral-line organs(10) and, more recently, in other somewhat similar experiments to be reported later. Comparison of normal and hypophysectomized *Rana pipiens* tadpoles from these experiments with respect to tail-tip regeneration only is presented here.

Materials and methods. The study is based on 113 tail-tip regenerations from repeated amputation in 29 *Rana pipiens* tadpoles. Sixteen of these animals were normal controls for 13 which had been hypophysectomized in the late tail-bud stage of the embryo by the standard procedure of Smith(11). Criteria used for successful hypophysectomy were the characteristic alteration in pigmentation and persistence of the larval state. Ages of both hypophysectomized and normal animals ranged from 3½ to 5 months at start of the series of amputations. Additional data were obtained from tail-tip amputation on 4 tadpoles which had been hypophysectomized one year and 9 months previously. Tadpoles were kept individually in finger bowls and fed maximally on desiccated liver and spinach. Tricaine methanesulfonate (MS222) anesthetization was used during each amputation and while the animals were in the microchamber for observation of the regenerating system. Tail-tips were measured by use of an ocular grid in the compound microscope. Serial sections of 12 regenerated tail-tips from control animals and 22 from hypophysectomized ones were prepared by standard Zenker-haematoxylin-tetrabromfluorescic acid technics.

Results. Tail-tips regenerated in all cases regardless of presence or absence of the epithelial hypophysis. The data from the first of the series of amputations are summarized in Table I. Not only did all tail-tips regenerate, but there is no significant difference in amount of regenerated tail-tip per unit time related to presence or absence of pituitary influence. The calculated index (mm/days) is not intended to imply a uniform *rate* of regeneration, but is a correction for the possibly misleading figure giving percentage of tail-tip regeneration by recognition of the various times allowed for regeneration. The timing was dictated, of course, by factors related to the primary purpose of the experiment–study of lateral-line organ regeneration.

Data from the second through the seventh set of amputations are summarized in Table II. There is again no essential difference between experimentals and controls. Furthermore, there seems to be no significant diminution in ability to regenerate tail-tip with increased age in the hypophysectomized animals or up to time of resorption of the tail at metamorphosis in the controls. Since constant temperature was not maintained during the course of the experiment which ran from time of hypophysectomy in October to the following September, effects of different seasonal temperatures are undoubtedly reflected in the data. However, at any given time during the experiment all animals were under

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TABLE I. Data from First Set of Regenerations.

Tail-tip amputated (mm)* H†	Tail-tip amputated (mm)* C†	Time of regeneration (days)	Tail-tip regenerated (mm)* H	Tail-tip regenerated (mm)* C	% of Tail-tip regenerated H	% of Tail-tip regenerated C	Index (mm/days) H	Index (mm/days) C
6.		51	5.		83.3		.10	
8.	6.5	47	5.5	7.	68.8	107.	.12	.15
	3.	47		5.5		183.		.12
	8.	43		4.		50.		.09
	8.	38		4.5		56.3		.12
4.	9.	36	4.	7.	100.0	77.8	.11	.19
6.	7.	36	5.	5.	83.3	71.4	.14	.14
4.	10.	36	4.	5.	100.0	50.0	.11	"
7.	8.	35	3.5	5.	50.0	62.5	.10	"
5.5	7.5	35	4.	5.	72.7	66.7	.11	"
7.	5.5	35	5.	5.	71.4	91.0	.14	"
5.	11.	35	4.	4.	80.0	36.4	.11	.11
	7.	35		5.		71.4		.14
	7.	35		4.		57.1		.11
	5.5	35		4.		72.7		.11
7.		34	5.		71.4		.15	
5.		34	3.		60.0		.09	
6.		34	4.		66.7		.12	
5.		34	4.		80.0		.12	
	5.	33		4.		80.0		.12
	8.	24		2.5		31.3		.10
					Avg index (mm/days)		.12	.13

* mm to nearest 0.5. † H = hypophysectomized tadpole; C = control.

similar conditions and may be reasonably compared.

The 4 animals hypophysectomized one year and 9 months prior to amputation also regenerated tail-tips. Approximately one-third of the amount removed was replaced in 20 days. One of the animals died before measurement, but the 3 others regenerated 5 mm, 4 mm, and 4 mm, respectively giving an average index (mm/days) of .22. These tail-tips regenerated in June-July under temperature conditions similar to those for series 5 listed in Table II. Histologically no essential differences could be determined in the characteristics of the regenerates between control and hypophysectomized animals.

TABLE II. Summary of Data from 2nd through 7th Set of Regenerations.

Amputation No. 2 H*	2 C*	3 H	3 C	4 H	4 C	5 H	6 H	7 H
mm regenerated/days								
.15	.20	.16	.22	.23	.31	.17	.23	
.17	.23	.21	.14	.23	.16			
.12	.17	.14	.18	.13	.16			
.15	.25							
.15	.21	.18	.19	.16	.19	.22	.12	
.17	.27	.14	.30	.10	.22			
.15	.14		.15		.19			
.14	.24	.21	.18	.23	.09	.35	.18	.13
.19	.19	.15	.24	.16	.31	.23	.23	.11
.18	.14	.16	.15	.16	.09	.25	.18	
.20	.25	.16	.24	.16	.13	.23	.18	.09
.15	.15	.20	.14	.26		.35	.20	.16
Avg index (mm/days)								
.16	.20	.17	.19	.18	.19	.26	.19	.12

* H = hypophysectomized tadpole; C = control.

Discussion. It appears obvious from the foregoing data that removal of the epithelial hypophysis in the embryo does not prevent regeneration of the tail-tip in the *Rana pipiens* tadpole. Twenty-nine cases from earlier experiments on regeneration of lateral-line organs in regenerating tail-tips of hypophysectomized tadpoles may be cited as additional evidence(10). Of these, 14 cases were *Rana pipiens*, 11 *R. sylvatica* and 4 *R. palustris*. The tadpole tail, therefore, differs strikingly from the Triturus limb in its independence of pituitary influence in regeneration.

Whether the cells of the blastema in urodele limb regeneration are dedifferentiated specialized cell types(4,5) or mesenchymatous fibroblasts(12) there is an aggregation of indifferent cells from which the new limb develops epigenetically. Holtzer(13) has presented evidence that the urodele tail also forms a blastema and that tail and limb regeneration may be more similar than formerly thought. If this should be true for the anuran tail, the well-supported hypothesis(6) that it is amputational stress which brings into operation a synergistic pituitary-adrenal action necessary for wound healing in preparation for blastema formation in the limb would not hold for the anuran tadpole.

Schotté and Murphy(7) suggest that the essential difference between limb and lens regeneration with respect to their differing responses to hypophysectomy is that, in contrast to limb amputation, there is no trauma to the iris in lensectomy. Since no wound healing in the dorsal iris precedes lens regeneration, presence of the pituitary is unnecessary. However, Stone(9) excised a dorsal segment of the iris concomitantly with lensectomy and obtained regeneration of lens from the wounded iris. Rate of regeneration was slower in hypophysectomized newts than in the controls but regeneration was completed.

Stone and Steinitz(8) attribute the fundamental difference between limb and lens regeneration to the different modes of origin of the cells forming the source of the regenerate. It is clear that the new lens has its origin in cells which result from the " 'dedifferentiation' of highly organized cells to a level at which they can be subjected to the inducing influence of factors directing them to a different aim"(8,p.493). Visual retina and iris also regenerate from retina pigment cells by a similar type of "dedifferentiation"(9). There is no true blastema formation.

It may be that the difference in response to pituitary absence in Triturus limb regeneration and anuran tail regeneration is genetic, or it may depend upon differences in the processes of wound-healing and blastema formation between limb and tail. In addition, it may be that different mechanisms are operating in adult and immature forms. A series of experiments to test the latter is in preparation.

Summary. 1. A comparison was made between regenerating tail-tips in normal and hypophysectomized *Rana pipiens* tadpoles. 2. No significant differences were noted in amount of regeneration/unit time or in the histology of regenerates. 3. Age of the hypophysectomized animal had no discernible effect on tail-tip regeneration. 4. Independence of pituitary influence in regeneration exhibited by the anuran tail and Triturus retina-iris-lens system is discussed in relation to the dependence of limb regeneration in Triturus.

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Glucuronide Formation of Narcotic Drugs *in vitro* and *in vivo*. (25631)

JULIUS AXELROD AND JOSEPH K. INSCOE (Introduced by S. S. Chernick)
Lab. of Clinical Science, Nat. Inst. of Mental Health, U. S. Dept. of H. E. W., Bethesda, Md.

A major pathway for metabolism of the narcotic drugs morphine(1,2), nalorphine(3), *l*-dromoran(4,5) and codeine(6) involves conjugation. Morphine(2) and *l*-dromoran(4) have been shown to be excreted as glucuronides, but the nature of "bound" codeine and nalorphine has not been established. The enzymatic mechanism whereby glucuronides are formed has been recently clarified. Dutton and Storey(7) found that o-aminophenol is conjugated with glucuronic acid by an enzyme in the microsomes of the liver that transfers glucuronic acid from UDP-glucuronic acid to the phenolic acceptor. This process has been shown to occur with many normally occurring and foreign phenols(8,9, 10), carboxylic acids(11) and amines(12) as acceptors. The formation of UDP-glucuronic acid is catalyzed by an enzyme that oxidizes UDP-glucose and requires diphosphopyridine nucleotide as the hydrogen acceptor(13). This paper describes *in vitro* formation of glucuronides of the narcotic drugs morphine, nalorphine, *l*-dromoran and codeine by an enzyme in the microsomes of guinea pig liver requiring UDP-glucuronic acid. In addition, excretion of glucuronides of morphine, nalorphine, *l*-dromoran and codeine by the guinea pig is shown.

Methods and materials. Preparation and assay of glucuronide forming enzyme. Adult male guinea pigs were killed, exsanguinated and the livers immediately removed and chilled in cracked ice. All subsequent steps were carried out at 0-5°C. The liver was homogenized with 4 volumes of isotonic KCl and the resulting homogenate centrifuged at 8000 x g to remove mitochondria, nuclei and unbroken cells. After separating the microsomes by centrifugation at 78,000 x g, they were washed with isotonic KCl and resuspended in this medium. Incubations were carried out at 37°C, using microsomes obtained from 1 g of guinea pig liver, 20 μmoles $MgCl_2$, 200 μmoles tris (hydroxymethyl) aminomethane buffer pH 8.0, 0.8 μmole UDP-glucuronic acid, 0.5 μmole substrate and water to make a final volume of 4 ml. To determine extent of enzyme activity aliquots of the incubation mixture were taken at 0 time and after 30 minutes of incubation, and amount of substrate remaining was measured. *Estimation of narcotic drugs.* Morphine and nalorphine were isolated from biological material by extraction into chloroform containing 3% isoamyl alcohol at pH 9.0 after saturating the aqueous phase with sodium chloride. The narcotic drugs were returned to 0.1 N HCl and measured using silicomolybdate reagent (14). Codeine(14) and *l*-dromoran were determined by procedures described previously (5). *Materials.* UDP-glucuronic acid was prepared enzymatically from UDP-glucose (Sigma Co.) and diphosphopyridine nucleotide by a procedure described previously(13). Bacterial β-glucuronidase was obtained from Sigma Chemical Co.

Results. Glucuronide conjugation of narcotic drugs in vitro. Incubation of morphine, nalorphine, *l*-dromoran and codeine with microsomes obtained from guinea pig liver and UDP-glucuronic acid resulted in disappearance of these compounds (Table I). In the absence of UDP-glucuronic acid, disappearance of these substrates was negligible. After 30 minutes incubation with UDP-glucuronic acid, 1 ml of pH 6.0 acetate buffer 0.5 N containing 500 units of β-glucuronidase was added to an aliquot of the reaction mixture and incubation was continued for 18 hours. Almost all of the narcotic drug that disappeared when incubated with UDP-glucuronic acid reappeared after treatment with β-glucuronidase. From these results it can be concluded that an enzyme present in guinea pig microsomes can transfer the glucuronic acid of UDP-glucuronic acid to narcotic drugs. The conjugation presumably occurs on the hydroxyl group.

Glucuronide formation of narcotic drugs in vivo. Guinea pigs were given morphine, nalorphine, *l*-dromoran and codeine intraperi-

TABLE I. Glucuronide Formation of Narcotic Drugs *In Vitro*.

Substrate	Substrate disappearing, μmole	Substrate reappearing after β-glucuronidase, μmole
Morphine + UDP-glucuronic acid	.31	.29
Morphine, UDP-glucuronide omitted	.06	.06
Nalorphine + UDP-glucuronic acid	.20	.19
Nalorphine, UDP-glucuronide omitted	.05	.05
Codeine + UDP-glucuronic acid	.18	.16
Codeine, UDP-glucuronide omitted	.03	.03
l-Dromoran + UDP-glucuronic acid	.36	.29
l-Dromoran, UDP-glucuronide omitted	.07	.07

Microsomes obtained from 1 g of guinea pig liver were incubated at 37°C with cofactors described under *Methods*. After 30 min. an aliquot of reaction mixture was removed for assay of substrate and 1 ml of pH 6.0 acetate buffer containing 500 units β-glucuronidase was added to another portion of reaction mixture. Incubation was continued for 18 hr and the mixture again assayed for substrate.

TABLE II. Excretion of Narcotic Drugs as Glucuronides in Guinea Pig.

Drug admin.*	Free drug excreted	Total conjugated drug excreted†	Drug excreted as glucuronide‡
		%	
Morphine sulfate, 90 mg/kg	7	39	38
Nalorphine sulfate, 90 mg/kg	5	12	12
l-Dromoran, 30 mg/kg	16	53	53
Codeine, 90 mg/kg	8	18	10

* Drugs given in 3 divided portions intraper. every 3 hr.

† Urine was autoclaved with one-tenth vol of concentrated hydrochloric acid at 115°C for 30 min.

‡ Urine (3 ml) was incubated with 25,000 units of bacterial β-glucuronidase, pH 6.0 for 24 hr.

toneally and the urine collected for 24 hours. Urine was examined for unchanged compound, drug liberated after acid hydrolysis and after treatment with β-glucuronidase (Table II). All the administered drugs were excreted as glucuronides. With the exception of codeine, all conjugated material was present in the urine as glucuronide. Codeine was excreted partly as a glucuronide and partly in another conjugated form.

Summary. Glucuronides of morphine, nalorphine, *l*-dromoran and codeine are formed by an enzyme in liver microsomes that can transfer glucuronic acid from UDP-glucuronic acid to the narcotic drug. Morphine, nalorphine, *l*-dromoran and codeine are excreted as glucuronic acid conjugates in guinea pigs.

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Preliminary Observations on State of Insulin in Human and Bovine Pancreas.* (25632)

Harry N. Antoniades, Albert E. Renold, Yves M. Dagenais† and Jurgen Steinke‡ (Introduced by George W. Thorn)
Protein Foundation Lab., Jamaica Plain, and Baker Clinic Research Lab., Dept. of Medicine, Harvard Medical School, Boston, Mass.

Previous reports demonstrated that blood insulin-like activity could be adsorbed on cationic exchange resin, in the sodium form, at physiological pH. This activity could then be eluted from the resin with dilute acids(1,2, 3). Since insulin is negatively charged at physiological pH, its adsorption on cationic exchange resin suggested the presence of an insulin complex in blood, insulin being subsequently freed from the complex during elution with acid. Control experiments demonstrated that crystalline insulin was not adsorbed on the same cationic exchange resin at physiological pH, when dissolved in presence of human albumin(1). Insulin-I^{131} added to blood serum similarly was not adsorbed on the same resin. It was further observed that the blood insulin complex eluted from resin with 0.1 N H_2SO_4 (pH 3) was biologically inactive when tested for insulin-like activity with the rat diaphragm assay. However, when the eluate was subjected to precipitation at pH 10, insulin-like activity toward rat diaphragm reappeared,§ suggesting removal of a basic protein component of the complex at that pH. Present studies on insulin in human and bovine pancreas indicate that a major fraction of insulin in pancreas, as in blood, is readily adsorbed on cationic exchange resin, in the sodium form, from homogenates prepared in 0.15 M sodium chloride, at pH 6.6 ± 0.04. The studies presented here suggest that a major fraction of insulin in pancreas, as in blood, may be in a complex form, possibly with basic proteins, the insulin complex being insoluble in saline at physiological pH.

Materials and methods. Dowex-50 cationic exchange resin (x8; x2) in the sodium form, has been employed. The resin was prepared as for routine blood collection by washing with distilled water, sodium chloride, sodium hydroxide and disodium phosphate, the final pH of 5 ml 0.01 M sodium sulfate over 1 ml resin beads being 6.6 ± 0.4. The following procedure was used for extraction of insulin activity from pancreas: Frozen human or bovine pancreas was homogenized and suspended in 5 to 10 volumes of 0.15 M sodium chloride solution with a Waring blendor at 2°C. One volume of Dowex-50-x2 or 2 volumes of Dowex-50-x8 exchange resin (sodium form) were added to the pancreatic suspension, and the mixture stirred for 5 minutes at 2°C. Supernatant fluid was removed from the resin which was repeatedly washed with cold 0.15 M sodium chloride. Elution of insulin-like activity from the resin was carried out at 2°C with 2 to 5 resin volumes of 0.1 N sulfuric acid (5 to 15 min.) followed by elution (5 min.) with one to 3 volumes of 1 N NH_4OH at 2°C. The combined eluates were neutralized, diluted with 10 to 100 volumes of bicarbonate buffer and assayed for insulin-like activity. Biological assays for insulin-like activity were carried out with the rat adipose tissue technic based on the effect of insulin on $C^{14}O_2$ production from glucose-1-C^{14} by this tissue(4). Anti-serum neutralization studies of the insulin-like activity were carried out with anti-insulin serum obtained by immunizing guinea pigs against bovine insulin according to the procedure of Moloney (5).‖ Resin eluates for paper electrophoresis were obtained with 0.1 N NH_4OH. The pH of the eluates was adjusted to 3.0 with

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† Recipient of a Research Fellowship, R. Samuel McLaughlin Fn., Toronto, Ont.

‡ Recipient of Research Fellowship from Am. Diabetes Assn.

§ Gundersen, K., personal communication.

‖ Insulin anti-serum was prepared by Drs. Werner Creutzfeldt and Paul Lacy and was made available to us through their courtesy and generosity.

1 N H_2SO_4, and the precipitate removed by centrifugation at 2°C. The supernatant fluid was brought to pH 9.5 with 1 N NH_4OH, and the solution stored overnight at 2°C. Precipitate was removed by centrifugation at 2°C, washed with dilute ammonium hydroxide and suspended in the minimum volume of cold distilled water. In the presence of 0.1 N H_2SO_4 (pH 3.0) the sulfate salt of the basic protein was water soluble, and samples of this solution were applied to paper for electrophoresis. Pater electrophoresis for detection of pancreatic basic proteins present in resin eluate concentrates was carried out at pH 7.6 (phosphate buffer, ionic strength 0.1) and at pH 4.5 (acetate buffer, ionic strength 0.1) on the Spinco model R, series D, paper electrophoresis cell, at room temperature.

Results. Table I summarizes results obtained to date. Although these values are preliminary, comparison with recovery obtained with the classic acid-alcohol extraction procedure suggests that resin-extractable insulin accounts for an important fraction of total pancreatic insulin. Ten ml of wet resin volume (Dowex-50-x2) were sufficient for adsorption of at least 85% of the adsorbable insulin present in 1 g of bovine pancreas (Table II).

The values obtained relate to insulin-*like* activity only, as measured with the rat adipose tissue technic. However, the following observations suggest that the insulin-like activity measured was indeed insulin: (1) Eluates obtained from resin exposed under identical conditions to homogenates of tissues other than the pancreas exhibited no insulin-like activity; (2) insulin-like activity could be demonstrated at dilutions as great as hundredfold in buffer, greatly reducing the likelihood of non-specific interference; (3) hypoglycemic activity of resin eluates has been established in 24-hour fasted rabbits on 4 separate occasions; (4) anti-insulin serum against bovine insulin neutralized the insulin-like activity extracted from bovine pancreas with the use of Dowex-50-x2 resin (Na+ form) after acid-alcohol treatment of the extracts; (5) insulin-like activity of the eluates was destroyed by incubation with 0.02 M reduced glutathione.

TABLE I. Insulin-like Activity Extracted from Pancreas with Cationic Exchange Resins (Na+ form) at pH 6.6 ± 0.4 (Units/100 g of Pancreas).

Pancreas	Exchange resin	Insulin-like activity* (units)
Bovine†	Dowex-50-x8	150
	”	162
	”	142
	Dowex-50-x2	240
	”	180
	”	300
Human	Dowex-50-x8	63‡
	Dowex-50-x2	234
	”	397

* Combined 0.1 N H_2SO_4 and 1 N NH_4OH eluates.

† The rather wide range within each species may in part be due to differences in fatty infiltration of pancreatic tissue.

‡ Pancreas from 37-year-old diabetic. This value is not reported to estimate total extractable insulin in a diabetic pancreas, but solely to demonstrate presence of the resin extractable insulin complex in such tissue.

TABLE II. Insulin-like Activity Extracted from 1 g of Bovine Pancreas during Repeated Treatment of Pancreas Homogenate with Dowex-50-x2 Resin, in Sodium Form (Each Treatment Carried Out with 10 ml Wet Volume Resin).

Resin treatment	Exp. I	Exp. II
	units	
1	>1.8	1.5
2	.12	.2
3	.06	.05
4	< .02	< .02

These findings suggest that the portion of pancreatic insulin activity extractable with cationic exchange resin under the conditions described exists in a complex form with other proteins, presumably basic proteins. Adsorption at near-neutral pH, as described here, should not be confused with adsorption of crystalline insulin (or insulin partially purified) on acidic cationic exchange resin in the hydrogen form, which has been observed at pH values below 5.0(6,7). At these low pH values insulin bears a positive charge and can be adsorbed on the cationic resin and subsequently eluted with citric acid(6) or ammonium hydroxide(7). Control experiments have shown that insulin-I^{131}, added to pancreatic tissue during its homogenization, is not adsorbed on the cationic exchange resin at physiological pH, indicating that the insulin complex is not the result of non-specific binding of insulin to basic proteins of pancreatic homogenates. Paper electrophoresis of resin

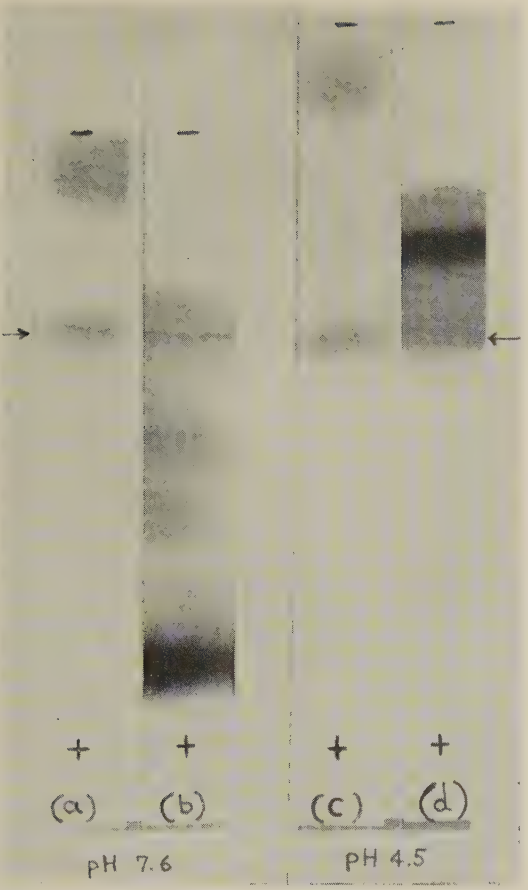

FIG. 1. Paper electrophoresis of pancreatic basic protein concentrate prepared from resin eluates, at pH 7.6 (phosphate buffer, ionic strength 0.1) and at pH 4.5 (acetate buffer, ionic strength 0.1). (a) and (c) basic protein concentrate; (b) and (d) plasma control.

eluates obtained from bovine pancreas indicates the presence of protein(s) which migrate as cations (Fig. 1). At 2 different pH values (pH 7.6 and pH 4.5) only a single basic protein appears on paper electrophoresis, although additional information will be needed before homogeneity of this protein fraction may be assumed.

Consistent with the presence of an insulin complex in pancreas is the observation that insulin-like activity present in resin eluates was not inhibited by addition of anti-insulin serum. Subsequent treatment of the eluate by acid-alcohol precipitation altered insulin-like activity of the supernatant in that neutralization of the activity by addition of anti-serum could now be obtained. This suggests that insulin present in the eluate was bound to other proteins which prevented its reaction with the anti-serum. Upon removal of these proteins by acid-ethanol precipitation, reaction with the anti-serum could proceed.

The physiological significance of these observations is unknown. Lindner(8) has previously reported that insulin immediately extracted from fresh beef pancreas under special conditions is combined with a basic protein of low molecular weight, probably a histone. The preparation, termed "Nativinsulin," is poorly soluble at the pH of blood and tissue and is but slowly adsorbed from subcutaneous injection sites. The major association of insulin with basic proteins in pancreas, postulated here on the basis of observations with cationic exchange resin, may primarily serve to allow for insulin storage at physiological pH.

Summary. A major fraction of insulin in human and bovine pancreas is readily adsorbed on cationic exchange resin, in sodium form, from homogenates prepared in 0.15 M sodium chloride at pH 6.6 ± 0.4. These studies suggest that this fraction of insulin in pancreas may be in a complex form, possibly with basic proteins, the insulin complex being adsorbed on the resin under specified conditions.

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In vivo Monoamine Oxidase Inhibition Measured by Potentiation of Tryptamine Convulsions in Rats. (25633)

David H. Tedeschi, Ralph E. Tedeschi and Edwin J. Fellows
Research and Development Division, Smith, Kline and French Labs., Philadelphia, Pa.

Evidence has been presented(1,2) that potentiation of the convulsant action of tryptamine serves as a useful measure of *in vivo* monoamine oxidase (MAO) inhibitory activity. Presumably, the mechanism of potentiation involves prevention of the destruction of tryptamine by MAO. Thus, doses of tryptamine which are sub-convulsant in normal animals because of rapid destruction of tryptamine by MAO, become convulsant in the presence of an MAO inhibitor. Biochemical evidence in support of this concept will be presented (Green and Sawyer, to be published). The present report is concerned with *in vivo* MAO inhibitory activity of a variety of pharmacological agents as measured by potentiation of the convulsant effects of tryptamine.

Methods. The following drugs were investigated: harmine, tranylcypromine (trans-2-phenylcyclopropylamine hydrochloride, SKF *trans* 385); SKF *cis* 385 (*cis*-2-phenylcyclopropylamine hydrochloride); *dl*-PIH (JB-516, α methyl-phenethylhydrazine); *d*-PIH, RO-4-1018 (*dl*-N-acetylmethionyl-isopropylhydrazide); nialamide [N-benzyl-3-(isonicotinylhydrazino) propamide]; phenelzine (β phenethylhydrazine); iproniazid (1-isonicotinyl-2-isopropylhydrazine); isonicotinyl-PIH (1 - isonicotinyl - 2 - phenylisopropylhydrazine dihydrochloride); diphenhydramine hydrochloride; tripelennamine hydrochloride; isoniazid (isonicotinic acid hydrazide); dihydroergotamine; mescaline; SKF 525-A (β-dimethylaminoethyldiphenylpropylacetate hydrochloride); SKF 5-A [2-amino-1-(3,4-methylenedioxyphenyl)-propane hydrochloride]; procaine; and procaine amide. A detailed description of the procedure for measuring potentiation of the convulsant effects of tryptamine has been reported(2) and is only briefly summarized here. Groups of rats were first treated with various doses of the drug under investigation and later, at time of peak drug effect, all rats were injected intravenously with 5 mg/kg of tryptamine hydrochloride. This dose of tryptamine causes convulsions in only 4% of untreated rats and is referred to as a CD_4. The dose of drug effective in causing 50% of rats to respond to the CD_4 of tryptamine with 3 seconds or more of uninterrupted clonic seizure activity (ED_{50}) and 95% fiducial limits was calculated according to the graphic log-probit method of Litchfield and Wilcoxon(3).

Results. A summary of tryptamine potentiating activity of the drugs investigated is presented in Tables I and II. Compounds in Table I effected significant potentiation and therefore permitted ED_{50} determinations. Compounds have been arranged in descending order of potency, subcutaneously administered harmine being the most potent and isonicotinyl-PIH least potent. The compounds in Table II failed to effect significant potentiation of tryptamine's convulsant effects. Diphenhydramine effected a maximum of 43% potentiation at a dose of 150 mg/kg; higher doses caused neurotoxic effects *per se*. None of the remaining compounds listed potentiated tryptamine, in spite of the fact that in most instances barely subtoxic doses of each compound were employed.

Discussion. All of the compounds found to be effective in potentiating the convulsant effects of tryptamine have also been reported to be potent *in vivo* inhibitors of MAO [harmine (4,5,6), tranylcypromine(7,8), SKF *cis* 385 (8), *d*-PIH(9), *dl*-PIH(10), RO-4-1018(11), nialamide(12), phenelzine(13), iproniazid (14) and isonicotinyl-PIH(15). In contrast, many of the compounds found to be devoid of tryptamine potentiating activity [Table II, also see(2)] have also been reported to be devoid of *in vivo* MAO inhibitory activity. It is concluded that the tryptamine potentiation potency of the compounds tested in this series reflects their potency as *in vivo* inhibitors of MAO.

Iproniazid has been used by most investi-

TABLE I. Potencies of Various Compounds as Potentiators of the Convulsant Effects of Tryptamine in Rats.

Drug*	Time of peak effect	ED_{50} (95% fiducial limits), mg/kg	Potency rating (iproniazid = 1)
Harmine (s.c.)	15 min.	.02 (.01- .04)	455
Tranylcypromine	14 hr	.18 (.13- .24)	55
SKF *cis* 385	14 "	.42 (.30- .60)	24
d-PIH	8 "	.56 (.34- .88)	18
dl-PIH	10 "	1.1 (.5 - 2.4)	9
Harmine (oral)	15 min.	4.0 (1.8 - 9.0)	2†
RO-4-1018	18 hr	4.4 (3.0 - 6.4)	2
Nialamide	18 "	5 (3.3 - 7.7)	2
Phenelzine	10 "	8.2 (5.1 -13.3)	1
Iproniazid	18 "	10 (6.3 -16.0)	1
Isonicotinyl-PIH	12 "	18 (14.6 -22.1)	.5

* Harmine was tested both orally and subcut. All remaining compounds were tested orally.

† Not significantly different in potency from iproniazid ($p > .05$).

gators as the standard for comparison of MAO inhibitors. Because of the wide variety of test procedures, species, route of administration, etc., employed by different investigators, it has not been possible to obtain from the literature an accurate comparison of potencies of the various inhibitors. With this aim in mind, relative potencies of effective tryptamine potentiators have been listed in Table I. Orally administered harmine was found to be approximately equivalent in potency to iproniazid, whereas subcutaneously administered harmine was found to be approximately 455 times as potent as iproniazid. This striking difference in potency is in general agreement with the findings of Tabachnick and Rubin(6) who reported that oral harmine (10 mg/kg, 1 hour pretreatment) failed to increase brain serotonin levels, whereas this dose of harmine administered intraperitoneally effected a significant increase in brain serotonin. The ED_{50} for oral harmine as a tryptamine potentiator was found to be 4 mg/kg. Attention is directed to the fact that time of peak effect for the ED_{50} was 15 minutes after drug administration. Significant potentiation of tryptamine was no longer detectable 1 hour after drug administration. It is conceivable, therefore, that the aforementioned investigators might also have detected a significant elevation of brain serotonin levels by the oral route had they employed a shorter pretreatment time.

Although relative MAO inhibitory potencies of drugs measured by the tryptamine potentiation test generally parallel their relative potencies by biochemical procedures, some differences were observed. For example, Horita(10) reported that *dl*-PIH was approximately 20 to 40 times as potent as iproniazid, whereas *dl*-PIH was only 9 times as potent as iproniazid as a tryptamine potentiator. Horita and Parker(15) indicated that isonicotinyl-PIH was approximately twice as potent as iproniazid, whereas in the present studies it was found to be only one-half as potent. Finally, Chessin *et al.*(13) found phenelzine to be approximately 8 times as potent as iproniazid, whereas the data presented here indicate that these compounds do not differ significantly in tryptamine potentiation potency. Differences in routes of administration, pretreatment times, species, etc., may largely account for these variations. However, one other factor should be considered. In the usual biochemical procedure employed for measurement of *in vivo* MAO inhibition, homogenization of tissues removed from animals pretreated with an MAO inhibitor may result in a greater interaction between inhibitor and enzyme system than may occur in the intact animal. Consequently, compounds which because of their physical or chemical properties would ordinarily come into relatively poor contact with MAO may appear to

TABLE II. Compounds Which Failed to Effect Significant Potentiation of Convulsant Effects of Tryptamine in Rats.

Compound	Time of test	Dose, mg/kg (oral)	% potentiation
Diphenhydramine	2 hr	150	43
Tripelennamine	½-3 "	100	0
Isoniazid	3 "	100	0
Dihydroergotamine	1-2 "	30(s.c.)	0
Mescaline	½-3 "	200	0
SKF 525-A	1-5 "	200	0
SKF 5-A	15 min.	15	0
Procaine	½-3 hr	100	0
Procaine amide	½-3 "	100	0

be more effective after homogenization, when such contact would be enhanced.

Costa(8) reported SKF *cis* 385 to be less active than the *trans* isomer as an *in vivo* inhibitor of MAO, although no quantitative data were presented. This finding agrees with the tryptamine potentiating potency of this compound; SKF *cis* 385 being approximately one-half as potent as the corresponding *trans* isomer. Biel and co-workers(9) have indicated that the dextro-isomer of PIH is approximately 50% more potent than the racemate *dl*-PIH as an *in vivo* inhibitor of MAO. Although *d*-PIH was found to be twice as potent as the racemate as a tryptamine potentiator, comparison of the respective ED_{50}'s revealed that this difference in potency was not statistically significant ($P > .05$).

Although Tickner(16) reported that diphenhydramine and tripelennamine were effective *in vitro* inhibitors of MAO, the *in vivo* data presented here do not support this conclusion. Diphenhydramine, for example, potentiated tryptamine only at barely subtoxic doses, whereas tripelennamine failed completely to potentiate tryptamine. It is concluded that these compounds as well as the other compounds listed in Table II are not selective *in vivo* inhibitors of MAO.

Summary. The *in vivo* monoamine oxidase (MAO) inhibitory potency of a number of drugs was tested by measuring their activity as potentiators of the convulsant effects of tryptamine. Effective MAO inhibitors, arranged in descending order of potency, included harmine (s.c.) tranylcypromine, SKF *cis* 385, *d*-PIH, *dl*-PIH, harmine (oral), RO-4-1018, nialamide, phenelzine, iproniazid, and isonicotinyl-PIH. Compounds ineffective as MAO inhibitors included diphenhydramine, tripelennamine, isoniazid, dihydroergotamine, mescaline, SKF 525-A, SKF 5-A, procaine, and procaine amide.

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Action of Digoxin and Insulin on Transport of Glucose through Myocardial Cell Membrane. (25634)

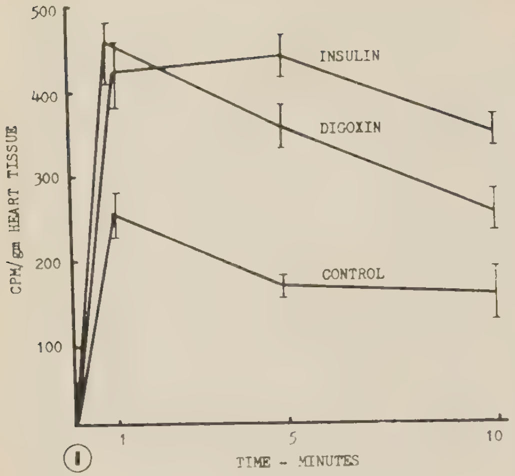
GERALD A. KIEN, ALLEN W. GOMOLL AND THEODORE R. SHERROD
(Introduced by K. R. Unna)
Dept. of Pharmacology, University of Illinois College of Medicine, Chicago, Ill.

A decrease in myocardial glucose utilization has been found to be associated with failure in the dog heart-lung preparation(1). Administration of glucose or insulin results in an increase in myocardial glucose utilization and a restoration of the competency of the preparation(2). Administration of lanatoside C to the failing heart-lung preparation has also been found to increase myocardial glucose utilization as evidenced by an increase in respiratory quotient of the cardiac glycoside-compensated preparation(3). Investigations with C^{14}-labeled substrates have demonstrated that cardiac glycosides increase rate of metabolism of glucose to carbon dioxide in the dog heart slice(4) and in the heart *in situ* (5). In view of the increased myocardial metabolism of glucose following administration of the cardiac glycosides or insulin, and the similarity of action of the cardiac glycosides and insulin on the dynamics of the failing heart-lung preparation, it appeared worthwhile to compare the action of digoxin with that of insulin on the rate-limiting reaction in utilization of glucose by the myocardium, *i.e.*, its transport across the membrane of the myocardial cell(6,7,8). This investigation deals with the effects of digoxin and insulin on rate of transport of galactose into the myocardium of the intact anesthetized dog. The criteria for the use of galactose as an indicator of glucose transport have been set forth by Levine (6).

Methods. The myocardial intracellular transport of glucose was determined by measuring rate of galactose-1-C^{14} entry and accumulation in the heart of normal dogs. Galactose-1-C^{14} used in these experiments had a specific activity of 0.88 μC/mM (Volk). Dogs weighing between 10 and 20 kg were anesthetized with pentobarbital sodium (35 mg/kg) in the post absorptive state, and allowed to respire room air supplemented with 100% oxygen *via* a nasal catheter. The external jugular vein was isolated and cannulated for administration of the drugs and isotopic substrate. Three groups of 6 animals were used in these studies. The first group received digoxin (0.065 mg/kg), the second group received insulin (1 unit/kg) and the third group served as the control. One-half hour after drug administration galactose-C^{14} was administered (0.5 μC/kg). The heart was quickly removed by a thoracotomy in the fourth intercostal space at 1, 5, and 10 minutes respectively, after administration of the isotope. The heart was freed of pericardium, fat and blood and homogenized in N perchloric acid (2 ml/g). Carbon dioxide liberated from the tissue was collected by means of a series of 4 gas washing towers, each filled with N sodium hydroxide. The carbon dioxide thus collected was precipitated as the barium salt and assayed for radioactivity. An aliquot of the total myocardial extract was assayed for radioactivity and the remainder was evaporated to dryness *in vacuo*. A sample of the dried extract, redissolved in a small amount of water was placed on Whatman #1 filter paper for chromatography in a butanol-formic acid-water(9) and a phenol-water solvent system(10). The chromatograms thus developed were placed on X-ray film for visualization of areas of radioactivity. R_f values thus obtained were compared with those obtained by chromatography of authentic galactose-1-C^{14}. Blood samples in the 3 groups were obtained from a femoral artery at time intervals of 1, 5, and 8 minutes following administration of the isotopic substrate. These samples were assayed for radioactivity as above. Statistical analyses of the data were made on the basis of a paired analysis of control and experimental groups throughout the time course of experiment.

Results. Fig. 1 illustrates myocardial galactose concentration (cpm/g) in control, insulin and digoxin-treated groups, as a function of time after intravenous administration of galactose-C^{14}. There was a significant increase in rate of galactose-C^{14} entry and accumulation in the myocardia of the digoxin-treated group ($P = 0.01$) and of the insulin-treated group ($P = 0.001$). There was no significant difference between myocardial galactose concentration in the digoxin and the insulin-treated animals. Integration of these curves revealed that administration of insulin or digoxin increased the extent to which galactose accumulated in the myocardium by 125 and 90%, respectively.

The myocardium did not metabolize galactose during the short duration of this experiment. There was no isotopic activity in carbon dioxide liberated from the heart tissue. Paper chromatography of the extract of the myocardium revealed, upon autoradiography, that all radioactivity on the developed chromatograms coincided with the R_f values of authentic galactose-C^{14}.



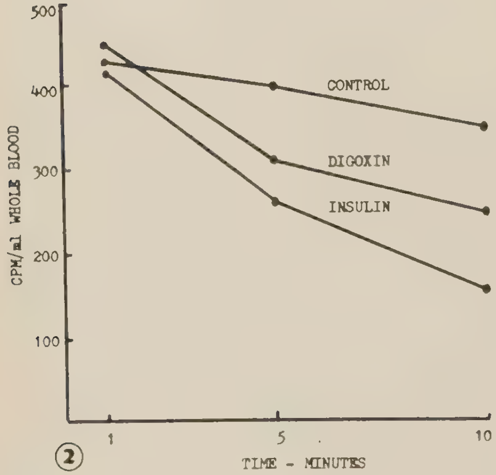


FIG. 1. Rate of accumulation of galactose-1-C^{14} in myocardia of control group, insulin-treated and digoxin-treated group, as a function of time following intrav. administration of labeled substrate. Each curve represents response in 6 dogs, 2 at each time interval. Perpendicular lines at each time interval represent range of deviation of the duplicate experiments.

FIG. 2. Rate of decrease of galactose-1-C^{14} in arterial blood as a function of time after intrav. administration of labeled substrate. Control, insulin-treated and digoxin-treated groups are represented by the mean responses in 3 dogs.

Fig. 2 illustrates rate of decrease of isotope activity in arterial blood of the 3 groups of animals. Digoxin increased rate of removal of galactose from the circulating blood volume. This has been observed in experiments with insulin(11). There was no significant difference between arterial concentrations of galactose-C^{14} in the 3 groups of animals at the one minute time interval. At the 5 and 10 minute time interval, however, there was significantly less galactose in the blood of the treated groups than the control ($P = 0.1$). There was no significant difference in galactose level between the digoxin and the insulin-treated group at any time interval.

Discussion. On the basis of conclusions drawn from experiments on galactose permeability with reference to the kinetics of glucose transport processes(6,7,8,11), these data indicate that digoxin and insulin augment transport of glucose into the myocardial cell. This effect of insulin is generally accepted as the mechanism whereby it increases the metabolism of glucose(12). The increase in myocardial galactose content following administration of digoxin may explain the earlier findings that the cardiac glycosides increase rate of metabolism of glucose by the myocardium(4,5).

Earlier experiments in this laboratory have shown that administration of therapeutic dose levels of digoxin increased rate of metabolism of glucose by the myocardium of the intact anesthetized dog and increased the flux of isotope through the cellular intermediates of the glycolytic pathway, the tricarboxylic acid cycle and the amino acids associated with these (5). These changes occurred in absence of changes in dynamics of the heart and hence without changes in metabolic requirements of the myocardium. Wollenberger(4) has demonstrated similar metabolic changes following administration of ouabain in the dog heart slice preparation. From these studies(5) it was concluded that the action of the cardiac glycosides appeared to augment one or more of the rate-limiting reactions of the glycolytic pathway of the myocardium, and in this manner, increased the contribution of cellular intermediates of the glycolytic pathway (*i.e.*, acetate, or oxaloacetate by carbon dioxide fixation) to the tricarboxylic acid cycle. This in turn, resulted in an increased rate of myocardial respiratory activity.

Burdette(13) has shown that administration of lanatoside C to human heart muscle slices resulted in an increase in oxygen consumption of the preparation and an increase in glucose uptake. Wollenberger(14) demonstrated that this respiratory stimulating effect of the cardiac glycosides in heart slices occurred only if lactic acid or glucose was present in the incubation media. Furthermore, this effect of cardiac glycosides could not be elicited if the heart tissue was homogenized. Thus, the presence of a functional cell membrane was necessary for the actions of cardiac glycosides.

Our experiments indicate that the increase in rate of metabolism of glucose to carbon dioxide (as observed in our earlier experiments) by the myocardium, subsequent to administration of the cardiac glycoside, is a function of an increase in transport of glucose (galactose) across the membrane of the myocardial cell.

Summary. Galactose transport into the myocardium of the anesthetized intact dog was measured as function of time following administration of either digoxin (0.065 mg/kg) or insulin (1 unit/kg). Administration of these drugs resulted in an increase in galactose entry and accumulation in the myocardium, thus indicating an increase in rate of glucose transport into this tissue. The data support the conclusion that digoxin increases myocardial glucose metabolism by facilitating entry of glucose into the myocardial cell.

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Effect of Neurohypophyseal Principles on Adenohypophyseal Activity in Toads. (25635)

C. BARKER JØRGENSEN AND LIS OLESEN LARSEN (Introduced by J. W. Everett)
Lab. of Zoophysiology A, University of Copenhagen, Denmark

It was shown that lysine-vasopressin injected into the common toad *Bufo bufo* (L.) was able to release hormone(s) from the pars distalis of the hypophysis(1), supporting the theory that vasopressin is chemically related to natural pars distalis stimulating factors(2). The present paper reports further investigation of the effect of neurohypophyseal principles on pars distalis function in toads. We were especially interested in determining minimal effective dose of vasopressin and in seeing whether the oxytocic principle was also able to stimulate the pars distalis.

Methods. In the toad, extirpation or inactivation of the pars distalis inhibits shedding of the slough, but not keratinization of the epidermis. Eventually, therefore, the skin becomes thickly cornified. The pars distalis stimulating activity of the preparations used could be evaluated from their effect on the skin. A preparation was judged capable of stimulating the pars distalis if it caused total or partial shedding of the slough within 24 hours after injection into toads with inactivated pars distalis, but not after injection into toads with extirpated pars distalis. Inacti-

TABLE I. Effect of Neurohypophyseal Principles on Shedding of Slough in Toads with Extirpated or Transplanted Pars Distalis of the Hypophysis.

		No. of cases of:		
	Dose (I.U.)	Sloughing	No sloughing	Pars distalis
Lysine-vasopressin, highly purified	.001	0	6	Transplanted
	.01	7	4	
	.1	14	1	
" synthetic	.1	8	0	"
" highly purified	.01	0	6	Extirpated
	2.0	0	3	
Insipidin AB (pressor preparation)	.001	3	2	"
	.01	4	0	
Syntocinon (synthetic oxytocin)	.1	0	6	Transplanted
	1.0	14	4	
Pitupartin AB (oxytocin preparation)	.1	0	5	"
	1.0	5	0	
Pitupartin AB	1.0	0	8	Extirpated
Extract of pars nervosa of whale*		3	0	Extirpated
Idem toad†		4	7	"
" "		2	7	Inactivated‡

* Extract of ½ mg acetone dried isolated pars nervosa of blue whale *(Balaenoptera musculus)* was inj. into each toad. Material was boiled with frog's Ringer for 5 min.

† Acetic acid extract of 1/10 of neuro-intermediate lobe of toad *(Bufo bufo)* inj. into each toad.

‡ Hypothalamic-hypophyseal portal circulation interrupted by extirpation of median eminence(1).

vation of the pars distalis was performed either by transplanting the gland into one of the eye muscles or by extirpation of the median eminence(3,1). The toads used were mostly males weighing 30 to 40 g. Injections were made of synthetic or highly purified lysine-vasopressin, of synthetic oxytocin (Syntocinon Sandoz), and of the pressor preparation Insipidin AB and the oxytocic preparation Pitupartin AB. Moreover some experiments were made with crude extracts of the pars nervosa of the blue whale (*Balaenoptera musculus* L.) and of the neuro-intermediate lobe of the common toad.* Generally the toads never shed the slough spontaneously after extirpation or inactivation of the pars distalis. However, some toads did slough once within a week after transplantation of pars distalis or after extirpation of the median eminence. As the transplanted or otherwise inactivated pars distalis is revascularized within a week of operation, this single shedding of the slough may be caused by substances leaking out from necrotic pars distalis tissue upon reestablishment of circulation. Each toad received from one to 4 injections, first about a week after operation. If sloughing followed injection, the next injection was not given until the skin was once more abnormally keratinized. Toads with extirpated or inactivated pars distalis survive for only a few weeks or months depending upon temperature and time of year. Before death the toads become increasingly weaker and cease to shed the slough in response to injections of pars distalis stimulating factors. The toads were therefore used only as long as they appeared healthy.

Results. Minimal effective dose of lysine-vasopressin is close to 0.01 international unit (I.U.) (Table I). Injection of this dose induced sloughing in 7 of 11 toads, whereas 0.001 I.U. was without effect. 0.1 I.U. caused shedding of epidermal cornification in all but one of the toads with transplanted pars distalis. As might have been expected, no significant difference was observed in effect of synthetic and highly purified lysine-vasopressin. 2 I.U. of purified lysine-vasopressin was without effect on the skin in the hypophysectomized toads confirming previous results that synthetic lysine-vasopressin has no direct effect on sloughing(1).

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* We thank Dr. Vincent du Vigneaud, Cornell University, for a gift of synthetic lysine-vasopressin, and Dr. Aa. Theil Nielsen, Royal Danish School of Pharmacy, for a gift of highly purified lysine-vasopressin.

Injection of the commercial preparation Insipidin caused sloughing even in hypophysectomized toads. The factor causing sloughing must have been present in high concentration, since amounts of Insipidin containing as little as 0.001 pressor unit were effective. The pars distalis stimulating effect of the vasopressin component of Insipidin could therefore not be observed.

A substance eliciting sloughing was likewise present in the pars nervosa of the blue whale and the neuro-intermediate lobe of the toad, since extracts of these glands provoked sloughing in hypophysectomized toads.

It is reasonable to assume that the "sloughing factor" that has been demonstrated in crude or partially purified neurohypophyseal extract is identical with the factor released from the pars distalis in response to vasopressin. This factor may be identical with adrenocorticotropic hormone (ACTH)(4) which is known to be present in significant amounts in the pars nervosa of the rat(5,6).

Injection of pars nervosa extract corresponding to 1/10 of a toad pars nervosa produced sloughing at least as often in the toads with extirpated pars distalis (in 4 out of 11) as in toads with inactivated pars distalis (in 2 out of 9). Thus, no additional effect on sloughing due to release of the sloughing factor from the pars distalis could be observed in the group with inactivated pars distalis. In separate experiments it was ascertained that inactivated pars distalis responded normally to injection of lysine-vasopressin. It can therefore be concluded that amounts of specific pars nervosa hormone(s) contained in 1/10 of a toad pars nervosa are not sufficient to cause sloughing by release of the sloughing factor from the pars distalis.[†]

Synthetic oxytocin and Pitupartin were capable of releasing the sloughing factor from the transplanted pars distalis, but larger doses than those of lysin-vasopressin were required to obtain an effect. Minimal effective dose was about 1 I.U., 0.1 I.U. being ineffective. Since Pitupartin is guaranteed to possess an activity of less than 0.02 pressor units for each oxytocic unit, the sloughing caused by injection of one oxytocic unit of Pitupartin may be due in part to presence of vasopressin in the preparation. Pitupartin apparently contained little or no sloughing factor as injection of 1 I.U. was without effect on the skin in hypophysectomized toads.

Discussion. In mammals, vasopressin is generally more effective than oxytocin in stimulating the inactivated pars distalis. In the rat minimal effective dose of synthetic vasopressin in releasing ACTH is about 0.1 I.U.(8). The rats weighed 300 g, or about 10 times as much as the toads used in the present experiments. Consequently, when compared on the basis of unit body weight the ACTH releasing mechanism in the rat and the sloughing factor releasing mechanism in the toad are about equally sensitive towards vasopressin. In the rat, vasopressin has been under consideration as the natural ACTH releasing factor(9,10). However, a closely related, but more potent peptide has been isolated from neurohypophyseal and hypothalamic tissue, so the ACTH releasing factor in mammals is probably not identical with the pars nervosa hormones, vasopressin and oxytocin(11,12,13). It would therefore be of interest to compare the effects of vasopressin and of the specific mammalian ACTH releasing factor on adenohypophyseal activity in toads.

Summary. In the toad *Bufo bufo* (L.) the pars distalis was inactivated by transplantation to one of the eye muscles or by extirpation of the median eminence. Minimal dose effective in releasing a sloughing factor from inactivated pars distalis was 0.01 I.U. of highly purified or synthetic vasopressin and 0.1 I.U. of synthetic oxytocin. Insipidin, a commercial preparation of vasopressin, and crude extracts of the pars nervosa of whale and toad contained large amounts of the sloughing factor which is probably identical with ACTH.

[†] Injection of extract of 1/1000 or less of toad pars nervosa suffices to produce antidiuresis in the toad(7).

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Biological Synthesis of a Growth Factor for Mammalian Cells in Tissue Culture.* (25636)

L. J. ALFRED AND R. W. PUMPER. (Introduced by R. J. Winzler)
Dept. of Microbiology, University of Illinois, Chicago

The search for simplified or chemically defined media has long occupied the efforts of those interested in growth of mammalian cells *in vitro*. Much of the recent reported work in this area has been done with the L strain of mouse skin fibroblasts isolated by Earle(1). Merchant and Rahn(2), Kuchler and Merchant (unpublished), and Waymouth(3) have shown the effects of peptone components on growth of L strain fibroblasts, and Gerarde and Jones(4) reported that bacto-peptone substituted for dialyzed embryo extract promoted collagen formation in chick embryo lung cultures. The chief difficulty encountered in preparation of defined media is that of replacing the serum which is usually present to the extent of 10-40% of volume. Lieberman and Ove(5) reported that a protein factor isolated from bovine serum stimulated growth *in vitro* of human appendix cells for short periods of time in presence of peptone-albumin, but in absence of whole serum. Recent experiments by Kutsky(6) have shown that a nucleoprotein isolated from embryo extract stimulated growth of chick embryo heart fibroblasts in cell cultures. Human cell culture lines have not as yet been adapted to growth in serum-free media, either chemically defined or containing peptone as a serum replacement. Preliminary experiments in our laboratory have shown that a stable cell line of mouse lung fibroblasts adapted to a serum-free medium by Pumper(7) release into the medium proteinaceous substance(s) capable of replacing the serum requirement of a human liver cell line isolated by Chang(8). Human liver cells were incapable of growing on the peptone-containing medium unless serum or protein from mouse lung cell growth fluid was added. Following this initial finding experiments were designed to separate the material responsible for stimulation of human liver cells from the dialyzable components of the growth fluid, and to study the physiological activity of the unpurified factor.

Methods. A. *Cell lines.* 1. Mouse lung cells adapted to a serum-free medium(7): Mouse lung cells were grown in milk dilution bottles on a medium consisting of 99% medium 199, 1 per cent Difco Bacto-peptone, 100 mg % dextrose, and 50 units each of penicillin and streptomycin per ml. The peptone was prepared in a 10% solution, and

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sterilized by autoclaving for exactly 10 minutes at 10 lb pressure. The pH was then adjusted to 7.2 with sodium bicarbonate. The cells were passed every 6-10 days by scraping them free from the glass with a bent tip pipette. The cell suspension was centrifuged and resuspended in complete medium to contain 3.0 × 10^5 cells per ml (hemocytometer count). These are now beyond their 104th serum-free passage. 2. Human liver cells: This cell line was isolated from normal human embryonic tissue by Chang(8). The cells were routinely grown on 90 per cent medium 199, 10 per cent horse serum, and penicillin-streptomycin (50 units each per ml), in T-60 Earle flasks. A normal inoculum consisted of 2.5 × 10^5 cells per ml, and the cells were passed every 6-7 days.

Results. Dialysis and lyophilization of growth fluid. As a preliminary step in separating the material responsible for the stimulation of the liver cells from salts and other dialyzable substances, the pooled growth fluid from serum-free mouse lung cells was dialyzed against running distilled water at 4°C for 72 hours, lyophilized, and stored at 4°C. An equal volume of uninoculated complete medium was treated in an identical fashion. The dry weight yield from uninoculated complete media was 1.8 mg/ml, representing the non-dialyzable portion of peptone + medium 199. The growth fluid, which was collected and pooled after maximum cell growth (6-10 days), gave a non-dialyzable fraction yield of 2.44 mg per ml. Both of these results represent mean values for 7 runs carried out on an original volume of 200 ml of fluid.

TABLE I. Effect of Heat on Stimulatory Activity of Material from Growth Fluid for Human Liver Cells.*

Added to medium 199	Treatment	Response (outgrowth) of liver cells after 24 hr
None	None	—
10% horse serum	”	+++
10% undialyzed fluid	56°C 0-45 min.	+++
Idem	” 60	—
10% dialyzed fluid	” 0-45	+++
Idem	” 60	—
Lyophilized material†	” 0-30	+++
Idem	” 45	++
”	” 60	—
”	100°C 5	—

* Washed human liver cells resuspended in medium 199 and used in a conc. of 1.0 × 10^5/ml.

† .5 mg/ml.

+++, max outgrowth compared to serum-containing cells. ++, poor outgrowth. —, absence of growth.

TABLE II. Growth Promoting Activity of Lyophilized Material from Serum-Free Mouse Lung Cell Growth Fluid for Human Liver Cells.*

Sample	Conc. lyophilized material, mg/ml	Response (outgrowth) of liver cells after 24 hr	Cell count after 48 hr, cells/ml × 10^5
Medium 199 only	.0	—	1.1
10% horse serum	.0	+++	4.0
Lyophilized material	.006	—	1.0
	.0125	—	1.8
	.25	++	2.0
	.5	+++	2.8
	1.0	+++	3.2
	2.0	+++	3.0
	2.5	+++	3.1

* Initial liver cell count = 1.0 × 10^5/ml.

+++, max outgrowth compared to serum-containing cells. ++, poor outgrowth. —, absence of growth.

Effect of heat on material from growth fluid. The effect of heat on the growth promoting activity of the material from growth fluid was determined, and the results are shown in Table I. It was shown that the substance responsible for stimulation of human liver cells in the absence of serum protein was stable to heat at 56°C for 45 minutes but rapidly lost the ability to support growth after this period. The material was coagulated at 100°C, and no stimulatory activity was detectable in the coagulum or supernatant fluid from this material. It was also shown that the stimulatory material from growth fluid was non-dialyzable and stable to lyophilization.

Stimulatory activity of lyophilized material from serum-free cell growth fluid. The stimulatory activity of the lyophilized materials is shown in Table II, which represents a typical experiment. The dried material was dissolved in balanced salt solution and varying concentrations were tested for physiological activity for human liver cells. Prior to stimulation experiments the liver cells were

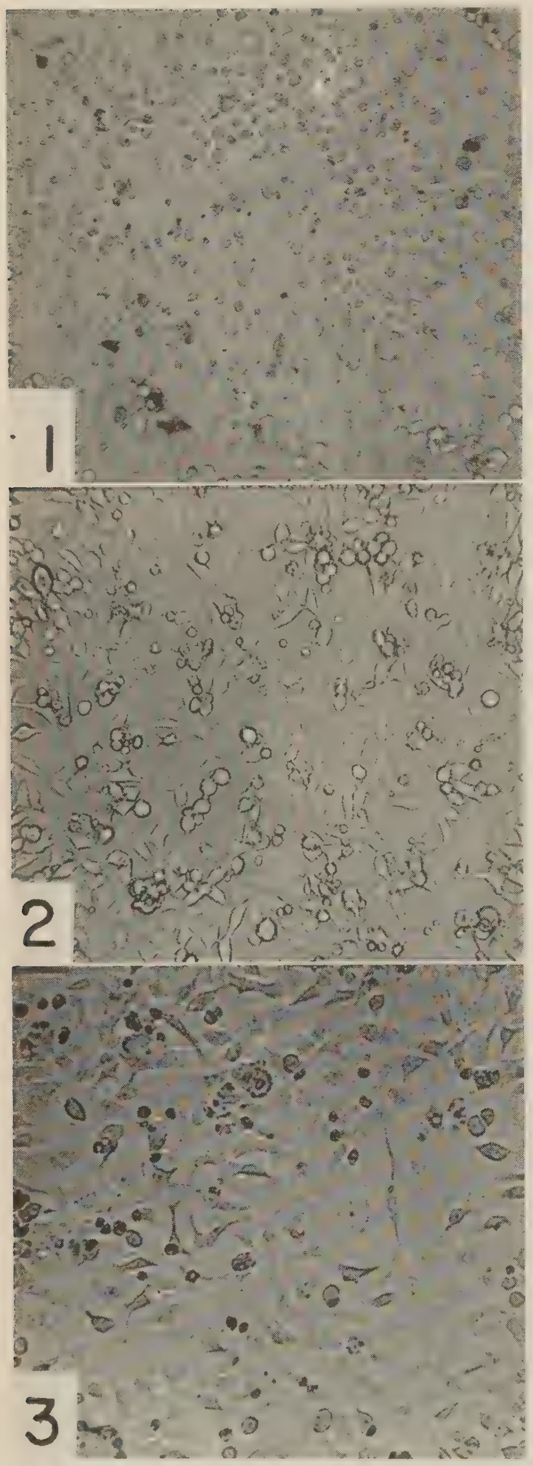

FIG. 1. 1. Culture of human liver cells grown on medium 199 only. × 100. 2. Normal culture of human liver cells grown on medium 199 + 10% horse serum. × 100. 3. Culture of human liver cells grown on medium 199 + lyophilized material from serum-free mouse lung cell growth fluid (conc. 0.5 mg/ml). × 100.

washed 3 times in balanced salt solution, once in medium 199, and counted with a hemocytometer. The stimulation was carried out in T-30 flasks, each of which contained medium 199, the sample material at varying concentrations, and 1×10^5 cells per ml. Observations for growth were made after 24 and 48 hours, and the cells trypsinized and counted after the 48 hour period. The results (Table II) show that the active substance present in the lyophilized material can replace serum protein in human liver cell cultures at least for short periods of time. It is further indicated that there is a graded response to the stimulatory factor. A series of photographs showing liver cell response to growth factor is shown in Fig. 1.

Discussion. This phenomenon was first observed when human liver and mouse lung cells were experimentally cultured on serum-free medium in the same flask. The human cells were able to grow very well as long as the serum-free mouse lung culture was present, but could not be propagated in its absence. Further attempts to grow human liver cells on a medium consisting of bactopeptone and constituents of medium 199 were unsuccessful. However, as shown in these studies mouse lung cells adapted to a serum-free medium(7) appear to synthesize a protein-containing substance that can be used for the growth of human liver cells in the absence of serum or its derivatives. Response of the liver cells to this factor appears to be quite similar to that obtained when 10% serum is added to the medium. There is a graded response proportional to concentration of material added.

Experiments are underway to purify and to determine the chemical nature and mechanism of action of the material produced in growth fluid of the serum-free mouse lung cell cultures.

Summary. A non-dialyzable substance was recovered in crude form from growth fluid of mouse lung fibroblasts adapted to a serum-free medium. This substance was capable of replacing the serum protein normally required for human liver cell growth. The substance in the material from mouse lung cell growth fluid is heat labile. The crude material was heat coagulable with loss of growth promoting activity for human liver cells. A graded dose response of growth of liver cells was obtained.

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Centrifugation of Rickettsiae and Viruses Onto Cells and Its Effect on Infection. (25637)

EMILIO WEISS AND HARRY R. DRESSLER
Virology Division, Naval Medical Research Inst., Nat. Naval Medical Center, Bethesda, Md.

Chick embryo entodermal cells have been extensively used in investigations of viruses of the psittacosis group and rickettsiae(1-4). During attempts to improve and simplify the basic procedure, it was found that centrifugation of the infectious agents directly onto the cells greatly enhanced infection. Preliminary results also indicated that the centrifugation technic might be profitably applied to other host cells. These studies were prompted by the report by Macdougald *et al.*(5) that chick embryo explants can withstand exceedingly high centrifugal forces and by the studies by Gey *et al.*(6) who effectively sedimented eastern equine encephalomyelitis virus onto HeLa cells.

Materials and methods. Microorganisms. The following strains were used: *Rickettsia prowazekii* (Madrid E), *Coxiella burnetii* (California), psittacosis virus (6 BC), and trachoma virus (TW 29, isolated in the laboratory of Dr. J. T. Grayston, Naval Med. Research Unit No. 2, Taiwan). Pools of these microorganisms were prepared from infected yolk sacs and diluted in Bovarnick's isotonic sucrose solution(7). Purified suspensions of typhus rickettsiae and trachoma virus were prepared by minor modifications of commonly used procedures(8,9) which included the following steps: 1. Treatment with trypsin ("Tryptar" Armour), 0.25% final dilution, for 1 hour at room temperature; 2. Centrifugation at 2,500 g for 45 minutes at 2°C in an angle centrifuge (Spinco); 3. Suspension of pellet in sucrose solution containing 0.6% bovine plasma albumin. The suspension was cleared with an amount of celite (Johns Manville) corresponding to 10% of weight of the yolk sacs and by centrifugation in an horizontal centrifuge at 500 g for 10 minutes at room temperature. The slightly opalescent supernatant was saved and used directly or subjected to step 4, centrifugation at 11,000 g for 20 minutes and suspension of pellet in tissue culture medium. Q fever rickettsiae were partially purified by a somewhat simpler procedure, while psittacosis virus was used as a crude suspension. All 4 agents were titrated in eggs, viruses by the routine serial 10-fold dilution method, rickettsiae by the single dilution method as described previously (9,10), or as explained in a later section on *C. burnetii*. *Entodermal cell cultures.* Explants were obtained from the avascular membranes of the 4-day-embryo as described previously (1-3). When the experiments did not require microscopic observation, explants were placed in standard round-bottomed 16 × 125 mm screw-cap tubes. The nutrient, 0.6 ml per tube, consisted of 20% rooster serum diluted in Hanks'(11) balanced salt solution. Tubes were incubated at 35°C in an upright position and monolayers were formed on lower surface by the 3d day. For microscopic observations the same procedure was used, except that explants were deposited on 12 mm round coverslips resting on the flat bottom of special tubes, prepared from standard 16 × 100 mm test tubes by Mr. A. D. Mack of Naval Med. Research Inst. Volume of the nutrient in these tubes was 0.5 ml. The cultures were usually inoculated on the 3d day after explantation by replacing the fluid with the inoculum diluted in fresh medium. To test the effect of centrifugation on infection, the cultures were centrifuged in a refrigerated horizontal centrifuge (International) for 1 hour. Because of the relatively long radial distance to the lower 5 mm of the tubes containing the cells and their fluids, centrifugal forces averaging 1600 and 1800 g were attained in the round- and flat-bottomed tubes, respectively. Temperature was maintained at 20°C during the centrifugation procedure or carefully matched to temperature of the control stationary cultures, which were maintained at 22-26°C. The cultures were then incubated at 35-37°C, depending on infectious agent used. The fluids were not changed during an experiment. For determination of total infectious titer of a group of cultures, the fluids were carefully removed and saved. The cells were disintegrated by rapid shaking for 1 hour at room temperature in a volume of 0.25% trypsin in isotonic sucrose solution equivalent to the nutrient. The cell brei, containing no microscopically recognizable cells, was added to the nutrient, and mixture was appropriately diluted in isotonic sucrose solution for titration. Coverslips were fixed in methanol and stained with May-Gruenwald-Giemsa. *Human skin cells* were prepared by Dr. N. H. Wiebenga, in our Division. They were obtained from the Tissue Culture Section, Lab. of Biology, Nat. Cancer Inst., N.I.H., and Tissue Bank Dept., Nat. Naval Medical Center. They were designated HuS 2806 and had been adapted to 10% horse serum in 10% NCTC 109 medium(12). Prior to their use in these experiments they were maintained for one month in a medium free of antibiotics. Following infection they were maintained on a medium containing only 2% horse serum.

TABLE I. Sedimentation of Typhus Rickettsiae Suspended in Tissue Culture Nutrient.*

	No. of infectious rickettsiae		
Rickettsial suspension	Upper	Lower	% in upper layer
	.3 ml		
Stationary	2.4 × 10^4	3.8 × 10^4	38.7
	2.6 "	1.8 "	59.1
Centrifuged	3.2 × 10^3	6.9 "	4.4
	3.9 "	16.2 "	2.4

* Rooster serum, 20%, in Hanks' balanced salt solution.

Results. Experiments with entodermal cells. R. prowazekii was used for some of the preliminary experiments. Table I presents results of a test of sedimentation of these microorganisms by the centrifugal force used in subsequent experiments. Purified rickettsiae were suspended in tissue culture medium and distributed in 0.6 ml volumes in screw-cap tubes, as in the routine tissue culture procedure. Six tubes were left in an upright stationary position, while another 6 tubes were being centrifuged, as described previously. The upper 0.3 ml of each tube was then carefully pipetted out, pooled in groups of 3, and titrated. The lower 0.3 ml was similarly treated. Rickettsiae remained approximately equally distributed in the 2 layers in the stationary tubes. In the centrifuged tubes the lower layer contained 95-98% of the rickettsiae, which would suggest that sedimentation was effective, but, possibly, not to the same extent as in the routine purification procedure.

Table II presents results of a similar experiment in which entodermal cell monolayers were used. Following centrifugation, or incubation at room temperature, the cultures were incubated for 1 hour at 35°C, to permit any additional interaction between microorganisms and host cells to take place. Nutrient fluids were then carefully removed and titrated, and the unwashed cells were broken

TABLE II. Sedimentation of Typhus Rickettsiae onto Entodermal Cells.

	No. of infectious rickettsiae		
Cultures	Medium	Cells	% with cells
Stationary	3.2 × 10^4	2.8 × 10^2	.9
	2.3 "	8.1 "	3.4
Centrifuged	1.2 "	2.3 × 10^4	65.7
	6.9 × 10^3	2.0 "	74.3

up and titrated separately. While over-all titers in the 2 groups of cultures were approximately the same, there was a pronounced difference in content of rickettsiae in the cell fractions, about 2% of the total in stationary and 70% in centrifuged cultures.

Table III presents results of a typical titration of typhus rickettsiae in entodermal cell cultures. Each of 6 ten-fold dilutions of rickettsiae were injected into 8 cultures, 4 were kept in a stationary position, and 4 were centrifuged. Cultures of each group were harvested at 7 days and titrated. In this and in several other experiments consistent infection of stationary cultures was not obtained with dilutions of rickettsial suspensions much greater than 10^{-2}. When the rickettsiae were centrifuged onto the entodermal monolayer, the cells appeared to be as susceptible as in the intact embryo. Experiments not shown in Table III indicated that peak titers were usually reached before the 7th day and numbers of rickettsiae seldom exceeded 3×10^5 per culture. Therefore, an increment was not clearly demonstrated in the group of cultures which had received the highest concentration of rickettsiae, but 100-200-fold increases were obtained in other groups (Table III). In some cases the cultures proved to be infected, but less than 3 rickettsiae per culture were recovered. This finding cannot be simply explained.

Some attempts to pass rickettsiae serially in stationary cultures failed, because, as appears from Table III, infection of the cells required large inocula, which, eventually, were not provided by the harvests of these cultures. By centrifugation, serial passage could be carried out without any particular difficulty or loss in rickettsial titer. The harvest from the 10th passage, which represented a 10^{-15} dilution of the original yolk sac, had a titer (confirmed by cross-protection test in cotton rats) of 2×10^4 rickettsiae per culture.

TABLE III. Recovery of Typhus Rickettsiae from Entodermal Cell Cultures.

Inoculum No. rickettsiae	Yield of infectious rickettsiae at 7 days (per culture) Stationary	Centrifuged
5×10^3	8×10^1	1×10^4
5×10^2	< 3	5 "
5×10^1	"	1 "
5	No evidence of infection	5×10^2
.5		6×10^1
.05		< 3

TABLE IV. Recovery of Q Fever Rickettsiae from Entodermal Cell Cultures.

	Effect on chick embryos			
	Deaths/total		Mean survival time*	
Time of harvesting (hr)	Stationary	Centrifuged	Stationary	Centrifuged
72	2/14	7/12	14.9	14.4
	3/14		14.8	
120	4/14	12/13	14.2	11.8
	8/14	15/15	13.4	12.3
166	12/14	13/13	12.4	11.5
	11/14	11/12	12.5	11.9

* Embryos surviving at 14 days were given 15 as day of death.

C. burnetii. Work with Q fever rickettsiae was complicated by the fact that they cannot be titrated by simple methods(13). Weiss and Pietryk(2) enumerated foci of infection formed on monolayers of entodermal cells. By application of this method to the current study, differences were not noted in number of foci of infection in stationary and centrifuged cultures. Table IV illustrates an experiment in which cultures were harvested at 3 intervals of time and the harvests diluted 1:10 and injected into eggs. The results, expressed in terms of relative numbers of embryos dying in each group, and their mean survival times, indicate that greater numbers of rickettsiae were harvested from the centrifuged than from the stationary cultures, especially at 120 hours. However, the differences are not as great as those obtained with typhus rickettsiae.

Psittacosis virus and related viruses, grown in entodermal cells, produce inclusion bodies which can be readily recognized with an 8 mm objective(1,4). Therefore, experiments with psittacosis virus were evaluated by microscopic examination of the cultures, and a number of observations were made that could not be accomplished with the rickettsiae. For example, examination of cultures fixed and stained during the first cycle of infection, at approximately 34 hours after inoculation, revealed that the inclusion bodies were more evenly distributed and of more uniform size in centrifuged than in stationary cultures.

Table V illustrates the difference in titers obtained in eggs and in entodermal cell cultures fixed and stained 88 hours after inoculation. The cultures listed as "shaken" were prepared in roller tubes with one flat side, just large enough to support an 11 × 22 mm coverslip. These cultures were maintained in a horizontal position and were shaken at 80 oscillations per minute for 1 hour at room temperature. This procedure has been shown to enhance infection(1), but did not prove to be superior to the stationary cultures used in these experiments. Entodermal cells were infected in centrifuged tubes almost as readily as in the intact embryo. The differences between stationary and centrifuged cultures (Table V) are significant, if some of the qualitative differences, noted above, are considered. For example, some of the stationary cultures listed as "infected" appeared to contain a single relatively small cluster of elementary bodies.

A more valid appraisal of the difference between stationary and centrifuged cultures is presented in Table VI. In this experiment the cultures were fixed at 34 hours and infected cells detected with an 8 mm objective were counted. The difference between the infected cell counts of the 2 types of cultures appeared to be approximately 100-fold.

Trachoma virus. Following successful cultivation of trachoma virus by Gordon *et al.* (14) in centrifuged entodermal cell cultures, a preliminary experiment was carried out to determine effect of centrifugation. The cultures were inoculated with a single dilution of virus and fixed and stained at 67 hours, at a time when inclusion bodies were most conspicuous and still represented the product of initial infection(14). Infected cells were not uniformly distributed in stationary or centrifuged cultures and far too numerous in the centrifuged cultures to provide more than a rough estimate of the difference between the 2 groups, which appeared to be at least 10-fold. In the centrifuged cultures, microscopic fields (covering an approximate area of 0.16 mm^2) containing 60-100 infected cells could readily be found. Equivalent fields in the stationary cultures contained 5-9 infected cells. Calculated ID$_{50}$ for centrifuged cultures appeared to be of the same order of magnitude as that for eggs ($10^{-4.6}$).

TABLE V. Titration of Psittacosis Virus in Eggs and in Entodermal Cell Cultures.

Dilution of yolk sac suspension	Embryos: Deaths/total	Cultures: Infected/total		
		Shaken	Stationary	Centrifuged
10^{-5}	10/10	6/6	5/5	6/6
10^{-6}	"	4/6	4/6	"
10^{-7}	"	0/6	3/6	"
$10^{\ 8}$	5/10			0/4
ID$_{50}$	$10^{-8.0}$	$10^{-6.2}$	$10^{\ 6.7}$	$10^{-7.5}$

TABLE VI. Titration of Psittacosis Virus by Infected Entodermal Cell Count Method.

Procedure	Cultures No.	Virus dil.	Count ± S.E.	Calculated ID$_{50}$*
(Egg)				$10^{-8.2}$
Stationary	4	$10^{-3.3}$	338 ±116	$10^{-6.1}$
	4	$10^{-4.3}$	85 ± 22	$10^{-6.4}$
	4	$10^{-5.3}$	3 ± 1	$10^{-5.9}$
Centrifuged	17	$10^{-5.3}$	298 ± 40	$10^{-7.9}$

* ID$_{50}$ was calculated on the basis that it represents approximately 0.7 particle.

Experiments with human skin cells. Suspensions of skin cells were inoculated with typhus rickettsiae, psittacosis and trachoma viruses, but only psittacosis virus appeared to be highly infectious. The effect of centrifugation on infection with this virus was tested in an experiment similar to that illustrated in Table V. The skin cells, 100,000 per 0.5 ml, were suspended in a mixture of nutrient medium and inoculum and placed in flat-bottomed tubes with coverslips. The skin cells formed monolayers on the coverslips and ID$_{50}$ were determined by microscopic examination of the cultures fixed and stained 88 hours after inoculation. The ID$_{50}$ for eggs, and for the centrifuged and stationary cultures were, respectively, $10^{-7.7}$, $10^{-7.2}$, and $10^{-4.6}$. Thus, centrifugation increased infection at least 100-fold and raised it to a level approaching that obtained in eggs.

Discussion. The variation in efficacy of the centrifugation procedure with the 4 infectious agents can in part be attributed to differences in thermostability. For example, with a thermostable agent, such as *C. burnetii,* centrifugation, possibly, only accelerates adsorption which will eventually take place in stationary cultures. In the case of a thermolabile agent, such as *R. prowazekii,* centrifugation plays the dual role of accelerating adsorption and, by this action, of preserving activity of a greater number of particles.

During development of the centrifugation procedure, a number of arbitrary choices were made which require further testing. For example, speed of centrifugation was the highest which could be conveniently employed, but the effect of higher or lower speeds has not been investigated. Temperature during centrifugation is another variable which was eliminated by selecting 20°C. This choice was made as a compromise to prevent both thermal inactivation of infectious agents and inhibition of host-parasite interaction.

The practical advantages of the procedure are obvious. It is simple and efficient and produces consistent results that closely resemble those obtained in eggs. It also offers an opportunity to separate adsorption from the other phases of intracellular growth of the infectious agents. Similarity of results obtained with 4 different agents and 2 unrelated host cells indicates that this method may have wide applications.

Summary. A method for effective inoculation of cultured cells with some rickettsiae and larger viruses is described. Chick embryo entodermal cell monolayers were prepared either in standard screw-cap tubes or, when microscopic examination was required, in flat-bottomed tubes containing 12 mm round coverslips. Following addition of inoculum diluted in fresh nutrient, the tubes were centrifuged horizontally at 1600-1800 g for 1 hour at 20°C. By this procedure infection of cells with *Rickettsia prowazekii* was greatly enhanced (about 10,000-fold) over that obtained in stationary tubes, and susceptibility of entodermal cells appeared to be as great in centrifuged tubes as in intact embryo. Good enhancement was also obtained with psittacosis (about 100-fold) and trachoma (at least 10-fold) viruses and moderate enhancement with *Coxiella burnetii.* The same procedure was successfully used to enhance infection of cells from human skin cell line (HuS 2806) with psittacosis virus.

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Effect of Cortisone on Liver Phosphorylase Activity.* (25638)

W. C. HESS, I. P. SHAFFRAN AND E. L. EVERITT
Georgetown University Schools of Medicine and Dentistry, Washington, D.C.

Liver glucose-6-phosphatase activity, in adrenalectomized rats, is increased by cortisone(1,2). While this could explain increase in blood sugar induced by cortisone, it would not explain increased liver glycogen also induced. Phosphorylase is required for synthesis of glycogen in the glycolytic pathway. The effect of adrenalectomy and subsequent cortisone injection on rate of phosphorylase activity in rat liver has been determined.

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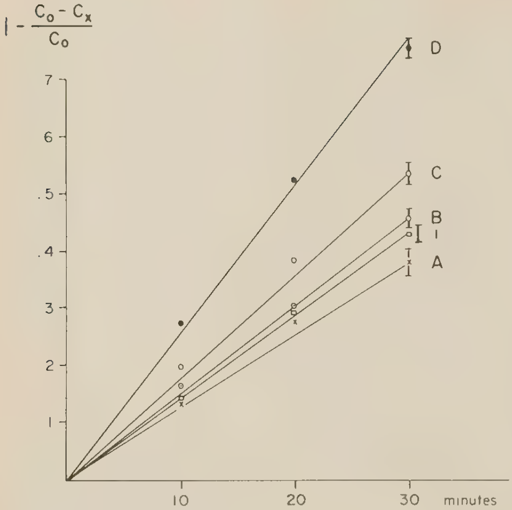

FIG. 1. Liver phosphorylase activity. C_o, mg P in substrate/mg N; C_x, mg P liberated/mg N; A. adrenalectomized, 1 intact control; B. 1.25 mg cortisone; C. 2.5 mg cortisone; D. 5 mg cortisone. Upper and lower limits of 30 min. values are given.

Methods. Intact and adrenalectomized female rats 125-150 g, fasted for 24 hours, of both Wistar and Sprague-Dawley strains, were used, 4-6 animals of each strain in each experiment. No strain differences were noted. Cortisone acetate, 1.25, 2.5 or 5 mg/100 g, was injected intramuscularly at the end of 24 hour fast and animals were sacrificed 24 hours later. Controls were sacrificed after a 48 hour fast. Livers were removed, analyzed immediately for phosphorylase activity by the method of Nimni *et al.*(3) and for glycogen by the method of Good *et al.*(4). Phosphorylase was determined after 10, 20, and 30 minutes for incubation. Activity was expressed as mg P liberated per mg N. Rate was determined by $1-\frac{C_o-C_x}{C_o}$ plotted against time, when C_o is mg P in substrate/mg N and C_x is mg P liberated/mg N.

Results. Fig. 1 shows liver phosphorylase rate changes induced by adrenalectomy alone and subsequent cortisone acetate injection. Average liver glycogen content of intact fasted controls was 0.14% and of adrenalectomized controls 0.10%. Average glycogens of adrenalectomized rats given 5.0, 2.5 and 1.25 mg cortisone acetate were 1.60, 0.68 and 0.31% respectively. Adrenalectomy decreased rate of liver phosphorylase activity below that of fasting intact animals. Cortisone increased rate of activity above that of controls; 1.25 mg gave a rate slightly, but not significantly greater, than that of intact fasted animals. Liver glycogen on the other hand, at this cortisone level, was significantly elevated over the fasting control.

Sorensen(5) reported a decrease in liver phosphorylase in fasted as compared with fed rats, both intact and adrenalectomized. Increases in liver phosphorylase activity were reported in obese hyperglycemic mice(6) and in rats during glucose absorption(3).

Since cortisone increases activity of both phosphorylase and glucose-6-phosphatase it probably also increased the activity of other enzymes that feed carbon chains into the glycolytic cycle. This would be necessary to produce an increase in both glucose and glycogen. Rosen *et al.*(7) and Gravasto *et al.*(8) found that hydrocortisone increases activity of glutamic-pyruvic and glutamic-oxalacetic transaminases respectively. These enzymes could provide, from protein, the necessary carbon chains for the glycolytic cycle since it is well known that peripheral protein is decreased by cortisone(9,10). Mark and Horecker(11) presented evidence that alternate pathways are present for glycogen synthesis but found that the glycolytic mechanism was the major one. One such pathway was recently discovered by Leloir and Cardini(12) who found a liver enzyme system that synthesizes glycogen from uridine-diphosphoglucose. The increases in phosphorylase and glucose-6-phosphatase activity may actually be secondary to transaminase activity and represent a response to increase in glucose precursors.

Summary. Rate of liver phosphorylase activity is decreased in fasted, adrenalectomized rats. Cortisone acetate administered at levels of 5, 2.5 or 1.25 mg/100 g body weight increased this activity above that of controls, the latter dosage only slightly above control level. Since glucose-6-phosphatase activity is also reported to be increased it is suggested that these 2 enzymatic effects may represent response to increased production of glucose precursors from peripheral protein.

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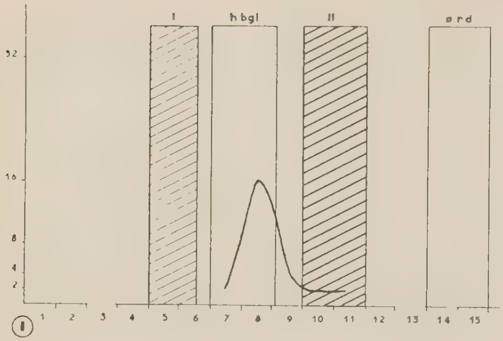
Zone Electrophoresis Studies on Hemagglutinin of Hemagglutinating and of Masked Strain of Polyoma Virus. (25639)

ROBERT CRAMER AND SARAH E. STEWART (Introduced by J. W. Beard)
Institut de Recherches sur le Cancer G. Roussy, Villejuif, Seine, France, and Nat. Cancer Inst., Bethesda, Md.

Eddy *et al.*(1) demonstrated an hemagglutinin in mouse embryo tissue culture fluids infected with polyoma virus. This hemagglutinin was lacking in cultures with no tumor-producing capacity. It was not possible to dissociate hemagglutination from the cytopathogenicity and tumor-inducing capacity by various methods such as heat treatment, ether treatment, limiting dilution titration, and first passage plaques from limiting dilutions. The authors, therefore, considered hemagglutination to be an inherent characteristic of the tumor-inducing agent. Kahler *et al.*(2) found in electron microscopic studies of purified polyoma virus an apparently uniform population of particles with a diameter of 40-45 mμ. By sedimentation experiments in a zonal density gradient particle counts were found to parallel the hemagglutination titer. However, no data on the relation of number of particles observed to possible tumor-inducing capacity are given. Hartley and Rowe (3) showed that culture fluids with high tumor-inducing capacity might fail to produce hemagglutination. It could be demonstrated, however, that unmasking of the hemagglutinin could be achieved either by heat or RDE treatment. It seemed desirable to study the interrelation of the different biological attributes of mouse polyoma issue culture fluids by methods other than sedimentation. Preliminary results of electrophoretic studies on the hemagglutinin both of hemagglutinating and of non-hemagglutinating (masked) Polyoma strains are given. Comparable studies on electrophoretic mobility of tumor-inducing capacity, and of cytopathogenicity are in progress.

Materials and methods. Virus. Strain 1000 with a hemagglutination titer of 1/128 and strain 8844 with a titer ¼ both from the Nat. Cancer Inst. were used. Both strains were known to give about 100% early tumor formation in Swiss mice. Virus was grown in 2% horse serum in medium 199. For unmasking the hemagglutinin of strain 8844, heat treatment for 30 to 60 min. in 56°C water bath was used. *Electrophoresis technic.* A detailed description of the electrophoresis apparatus and technic has been given(4,5,6). Tissue culture fluids were treated with one cycle of centrifugation for 10 min at 6000 g in a Spinco rotor 40. The supernate was dialysed against the buffer used for electrophoresis and mixed with the appropriate quantity of cane sugar to fit the density conditions for introduction into the electrophoresis column. Veronal buffer of pH 8.6 containing 205 g sodium diethylbarbiturate and 28 g diethylbarbituric acid in 10 l of distilled water was



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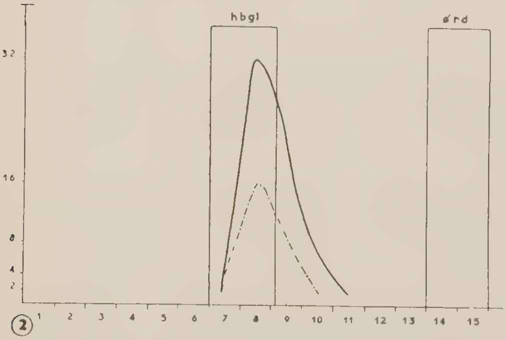

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FIG. 1. Zone electrophoresis diagram showing distribution of Polyoma hemagglutinin activity of strain 1000 (continuous line). Ha titer indicated on ordinate, consecutive electrophoretic fractions of 0.5 ml each on the abscissa. Reference substances rabbit hemoglobin (hbgl) and neutral red (ørd). Impurities: opalescent (I) and turbid zone (II).

FIG. 2. Electrophoresis diagram indicating mobility of hemagglutinin of strain 1000 (continuous line) and of unmasked strain 8844 (broken line) in relation to mobility of rabbit hemoglobin.

used. Electrophoresis was allowed to proceed for 2 to 3 hours at room temperature with a voltage gradient of 3.5 V/cm and a current of 15 m A. Experiments with and without addition of reference substances were made. Rabbit hemoglobin and neutral red served as reference substances. After completion of electrophoresis, the contents of the column were run out by a separate sampling capillary and divided into fractions of 0.8 ml or less. The hemagglutination titer of each fraction was tested by the pattern method(7), using 0.3 ml from each fraction added to 0.3 ml of a suspension of 0.25% guinea pig erythrocytes.

Results. Invariably 2 visible zones of "impurities" appear in all experiments (Fig. 1). A first opalescent zone (zone I, Fig. 1) migrates definitely slower than rabbit hemoglobin and separates from it. A second very sharply defined zone of turbidity (zone II, Fig. 1) appeared above the hemoglobin zone, equally well separated. A minor turbid zone not indicated in Fig. 1 migrated slightly more slowly than neutral red.

No major hemagglutination activity was found corresponding to either of these zones described. The major peak of hemagglutination appeared invariably between zone I and zone II, a zone occupied by rabbit hemoglobin in the experiments in which this substance had been added as reference substance.

As the hemagglutinin assumed the same position relative to zone I and zone II in experiments without addition of rabbit hemoglobin, the mobility recorded seems to be characteristic for polyoma hemagglutinin.

About 50 to 70% of hemagglutinating activity was recovered in each electrophoresis experiment.

It is evident that major impurities appearing in crude preparations can be separated by zone electrophoresis from the hemagglutinin; these impurities must correspond to undefined substances, probably released at the moment of cell lysis in the culture fluid. A part might be due to the 2% horse serum added to the original culture medium.

It was intended to compare mobility of the hemagglutinin of strain 1000 to mobility of the hemagglutinin of the masked strain 8844. According to Hartley and Rowe, a certain extent of unmasking could be achieved by heat treatment. In our experiments, exposure at 37° for 30 min was ineffective whereas with exposure at 56° for 30 to 60 min a certain extent of unmasking could be achieved with strain 8844. A remasking occurs when the sample is stored at 4°, which confirms the findings of Hartley and Rowe.

The hemagglutinin of the unmasked 8844 strain assumed the same position in the electrophoresis column as hemagglutinin of strain 1000 (Fig. 2). As electrophoresis was not extended over more than 3 hours, possible minor differences in electrophoretic mobility could have escaped observation. The relatively short time of electrophoresis was compensated by increasing inspection density *i.e.*, in sampling as small volumes as are compatible to test the hemagglutination. This seemed justified in sugar gradient zone electrophoresis because of the great stability against convection offered in this system.

Some electrophoresis experiments were done to test the possibility that a definite virus-inhibitor dissociation could be achieved in zone electrophoresis. For unmasking strain 8844, treatments with potassium periodate or RDE that would destroy or inactivate the inhibitor were avoided. The reason for this is that only preparations in which the inhibitor is preserved can be used for testing the possibility that a definite hemagglutinin-inhibitor dissociation may be achieved by zone electrophoresis. For this purpose, in some experiments, zone I (Fig. 1) of opacity was carefully removed by the first capillary that served to introduce the sample into the electrophoresis column. The hemoglobin zone containing the hemagglutinin was then removed separately by a second capillary, care being taken to finish sampling at a moment when the lower boundary of zone II (Fig. 1) just appeared above the tip of the second capillary. The hemagglutination titer of the purified hemagglutinin fraction was tested at once. At the same time, the hemagglutinin titer of the control sample of unmasked 8844 strain that had been used in electrophoresis but had been kept at the temperature of the room where the experiment was performed, was likewise tested for hemagglutination. To test the remasking effect, an aliquot of unmasked, not electrophoresed and unmasked "purified virus" was kept for 24 or 48 hours at 4°. After this time, both were again tested for hemagglutination using the same guinea pig erythrocytes in the same concentration.

Provided the stability of the hemagglutinin in the purified state is the same as observed in crude preparations, a comparison of hemagglutination titers of control and electrophoretically purified hemagglutinin before and after remasking treatment should indicate whether or not an inhibitor-hemagglutinin dissociation can be achieved by zonal electrophoresis.

One such experiment gave the results indicated in Table I.

TABLE I. Unmasking of 8844 Strain by Heat Treatment and Effect of Electrophoresis on Remasking Effect.

	HA titer			
8844	Before unmasking	After unmasking	Before remasking at completion of electrophoresis	After remasking
Control	1/8	1/64	1/64	1/8
Electrophoresed	1/8	1/64	1/32	1/32

The masked strain 8844 was used. For this strain, obtained from the Nat. Cancer Inst., a HA titer of ¼ was found. When the sample was retested one month later after having stayed at −70°, a titer of ⅛ was recorded. It is not clear if during this time some inhibitor action was lost. After heating at 56° for 60 min., an unmasking was found, the HA titer being 1/64. A remasking of the control sample was observed after treatment at 4° for 24 hours. No remasking was observed with electrophoresis fraction 8, as the same HA titer of 1/32 was observed whether the fraction was tested immediately after completion of electrophoresis or after having been for 24 hours at 4°. The possibility has been considered that the absence of remasking of purified electrophoresed virus might be due to the saccharose environment of the electrophoresis column rather than to a definite virus-inhibitor dissociation. Therefore, virus was added to saccharose in veronal buffer to give a final dilution of 20% and an aliquot was added to the same amount of veronal buffer. An initial titer of 2 was found. After heating for 1 hour at 56°, unmasking to a titer of 64 occurred. In both the saccharose and the veronal samples remasking to a titer of 2 occurred after staying at 4° for 24 hours. Another cycle of unmasking and remasking with the 2 samples gave the same result. The saccharose environment, therefore, can not be responsible for the unmasking. A definite dissociation between the hemagglutinin and its inhibitor by electrophoresis seems to be the most plausible explanation.

Summary. 1) Polyoma virus hemagglutinin of a hemagglutinating strain 1000 and of a masked strain 8844 were examined by zonal electrophoresis in a density gradient of cane sugar. 2) The masked strain 8844 was un-

masked by heat treatment previous to electrophoresis. 3) Both hemagglutinins migrated with the same speed slightly faster than rabbit hemoglobin when tested after an electrophoresis for 3 hours at pH 8.6. 4) Support for the presence of an inhibitor of hemagglutinin in the masked strain 8844 was gained. 5) It appeared that a hemagglutinin-inhibitor separation could be achieved when previously unmasked hemagglutinin was purified by electrophoresis.

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Demonstration of Immune Response to Foot-and-Mouth Disease Vaccine In Protection Test in Young Adult Mice. (25640)

RAYMUNDO G. CUNHA (Introduced by M. E. Wegman)
Pan American Foot-and-Mouth Disease Center, Rio de Janeiro, Brazil*

Since its development in 1938, aluminum hydroxide adsorbed foot-and-mouth disease virus vaccine(1,2) has gained wide acceptance throughout the world. Potency tests of this vaccine are generally conducted in cattle, the number used varying according to the quality of the test(3,4,5). The high cost of the test and the difficulty in infected countries of obtaining susceptible cattle generally make impracticable the potency testing of each batch of vaccine. It would be highly desirable, therefore, to find a laboratory animal that could be used as a substitute for cattle in such tests. Recent publications show that this problem is being investigated elsewhere. Uhlmann and Traub(6) refer to use of suckling mice to determine immune response of vaccinated adult mice and Skinner(7) refers to use of adult mice, the immunity of which is tested by antibody titration in suckling mice (as cited by Henderson,5). The experience that foot-and-mouth disease virus could be adapted to adult mice(8), led to investigation of the immune response of these animals to aluminum hydroxide adsorbed vaccines, assessing the immunity established by response to inoculation of the vaccinated mice with adapted strains of virus. Brief reference to this work has already been made(9) and in the present paper a description is given of experiments performed to evaluate the protection produced in young adult mice by foot-and-mouth disease vaccines prepared from virus of different sources and to determine if the protection afforded was type specific.

Materials and methods. A Rockefeller Institute strain of Swiss mice was used. Those referred to as suckling mice were 6 to 8 days old. The young adult mice were weaned mice, 3 to 6 weeks old, weighing 9 to 13 g at beginning of experiments. Older mice, up to 8 weeks and 21 g weight, were also used for certain preliminary experiments with type O vaccines. Aluminum hydroxide formalised vaccines were used that had been prepared in the State Veterinary Research Inst., Amsterdam, Holland; the Veterinary Research Inst., Maracay, Venezuela; the Animal Biol. Inst., Rio de Janeiro, Brazil or in Pan American Foot-and-Mouth Disease Center. The virus for vaccine preparation was obtained by the Frenkel method of culture(10), by inoculation of the tongue of cattle or by infection of

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TABLE I. Inoculation of Young Adult Mice with Foot-and-Mouth Disease Vaccine and Challenge of Immunity Produced with a Virus Strain Adapted to Young Adult Mice.

Vaccine		Mortality of mice inoculated with dilutions of type A adapted virus								
Type and batch	Dose, ml	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	Virus 50% end-point	Protection afforded in ID_{50}
A 4C*	.05	6/8†	5/8	7/8	4/8	3/7	0/7		$10^{-3.7}$	$10^{2.1}$
	.1	7/8	"	6/8	2/8	1/7	"		$10^{-3.3}$	$10^{2.5}$
	.2	5/8	"	5/8	3/8	2/8	2/8		"	"
	.4	3/8	0/8	2/8	3/8	1/8	1/8		$10^{-1.5}$	$10^{4.3}$
	Nil	8/8	8/8	8/8	7/8	7/8	4/8	0/8	$10^{-5.8}$	

10^{-1}, 10^{-2}, 10^{-3} dilutions of virus infected all mice in control groups, data from corresponding vaccinated groups are used to calculate 50% protection dose of vaccine, thus

Dose of vaccine	Log dose of vaccine	Protected	Not protected
.4	1.6	19	5
.2	1.3	9	15
.1	1.0	6	18
.05	2.7	6	18

By method of Reed and Muench 50% Protection Dose (PD_{50}) is calculated to be 0.2 ml.

* C—monolayer culture virus.

† No. dead/No. inoculated.

monolayers of bovine kidney cells (see Tables). Mice were vaccinated by the subcutaneous route in groups of 50 for each dose. An equal group of unvaccinated mice was set apart in each experiment for later use as controls. Twenty-one days later, 6 dilutions, 10^{-1} to 10^{-6}, of the challenge virus were inoculated intraperitoneally, 8 mice of the group of 50 being used for each dilution. Control groups received dilutions from 10^{-1} or 10^{-2} to 10^{-7}. The challenge virus was also titrated in suckling mice as a control of the infectivity of the preparation. These adapted viruses were from the 37th to the 49th passage of a type Vallée O strain, from the 89th to the 92nd passage of a Vallée A strain and from the 28th to the 34th passage of a type Waldmann C strain of foot-and-mouth disease virus in young adult mice. Adaptation was achieved by use of mice previously treated with 5 mg cortisone acetate for the earlier passages. The O and A strains were inoculated in doses of 0.25 ml and the C strain, 0.5 ml except for titration in suckling mice when for all strains the dose was 0.05 ml. The mice were observed for a period of 15 days, titration end-points were calculated by the method of Reed and Muench(11) and the difference between end-points obtained in vaccinated and in control mice provided an indication of degree of protection produced. The data of these titration results were also used to calculate the 50% protection dose (PD_{50}) of the vaccines by the accumulation method of Reed and Muench(11). This was done by noting the mice "protected" and "not protected" in the vaccinated groups inoculated with the dilutions of virus that had infected all the control mice. The result of a typical experiment is given in detail in Table I as an example of the methods employed and to facilitate interpretation of the subsequent tables.

Results. Preliminary experiments were performed with type O vaccines at a time when only the type O strain of virus had been adapted to adult mice. Two vaccines from the Animal Biol. Inst., Rio de Janeiro, and 2 from the State Vet. Research Inst., Amsterdam, were tested in a total of 7 experiments using mice from 5 to 8 weeks old. The results of these tests showed that foot-and-mouth disease vaccine, inoculated by the subcutaneous route in mice, conferred protection against the challenge virus and that this protection increased with the dose of vaccine. With the largest doses tested of these vaccines, 0.5 ml, virus titration end-points were lowered by 4 log units.

Subsequently, after type A and type C viruses were adapted to young adult mice, the investigation was extended to cover monovalent vaccines of the 3 virus types occurring in South America. The specificity was also in-

TABLE II. Inoculation of Young Adult Mice with Foot-and-Mouth Disease Vaccine and Challenge of Immunity Produced with Virus Strains Adapted to Young Adult Mice. Summary of results.

Vaccine		Titer* of challenge virus in control mice	Protection afforded in log ID_{50} to mice inoculated with vaccine doses (ml)				PD_{50} calculated as in Table I, ml
Type and batch	Log ID_{50} per ml		.05	.1	.2	.4	
O 1F†	5.3	5.0	Nil	Nil	Nil	1.5	.38
2F	7.2	4.6	”	”	”	2.1	.29
3F	7.6	4.5	1.7	.9	2.8	2.5	.27
VE‡		4.5	1.4	2.0	3.5	>3.5	.13
A 2F	6.5	4.6		2.0	3.2	3.4	.16
3F	6.5	6.6	3.9	4.2	3.7	5.0	.17
4F	6.3	6.1	2.9	2.9	3.9	3.1	.14
5F	7.0	5.3	2.5	3.3	2.7	4.5	.10
6F	7.1	5.3	1.8	.7	1.9	2.7	.34
VE		4.5	1.9	2.5	3.2	3.3	.14
C 1F	7.1	5.2	3.9	3.4	>4.2	>4.2	.13
3F	7.5	4.0	2.6	2.6	3.0	>3.0	.07
1C§	7.4	4.0	>3.0	>3.0	>3.0	”	<.05

* Negative powers of 10.

† F—Frenkel culture virus.

‡ VE—Tongue epithelium virus, Venezuelan vaccine.

§ C—Monolayer culture virus.

vestigated of the immunity conferred by a monovalent vaccine. Experiments were performed with monovalent vaccines of types O, A and C by vaccinating groups of young adult mice with doses of 0.05 ml, 0.1 ml, 0.2 ml and 0.4 ml. Except for 2 vaccines received from the Vet. Research Inst., Maracay, all the remainder were prepared in this Center's Laboratory for other experimental purposes and the number of ID_{50} of virus contained in each ml of vaccine was known (Table II). These data were obtained by titration of virus suspensions in suckling mice, except for vaccine A 4C (Table I), the titration for which was done in bovine kidney monolayer tubes. Table II summarizes results obtained with types O, A and C vaccines against virus of the homologous immunological type.

To check that protection induced by a monovalent vaccine was specific only to the homologous type of virus, 5 experiments were carried out with vaccines of different types. Only one dose of vaccine was used but 3 groups of 50 mice were inoculated, one group for each type of challenge virus. The results obtained are shown in Table III, and indicate that the protection induced by a monovalent vaccine is type specific.

In Table III the results have been included of titration of the challenge virus in suckling mice. These results are similar to those obtained in corresponding titrations performed in connection with the experiments recorded in Table II. Although the strains of virus adapted to adult mice regularly produce infection in this host over a sufficient range of dilutions to make their use practicable in this type of experiment, infectivity of the strains is still much greater for suckling mice.

Discussion. The present experiments have shown that foot-and-mouth disease vaccines induce immunity in young adult mice, this immunity being checked by direct inoculation of vaccinated mice with virus strains adapted to adult mice. There is no doubt that this protection test is more practicable than those technics(6,7) proposed in which the additional use of suckling mice is required. The results obtained by comparing the differences in virus titration end-points in vaccinated and in control mice showed considerable irregularity in correlation between dose of vaccine and grade of immunity although there was a definite trend towards larger doses giving better immunity. Application of accumulation technic of Reed and Muench in assessing the 50% protection dose had the effect of removing much of this irregularity of response. The experiments were not originally designed for interpretation of the results in this qualitative manner. Further investigation of this point will be necessary before deciding whether potency of the vaccine should be expressed in terms of ID_{50} protection in the manner of the Habel test of rabies vaccine(12) or in terms of the dose of vaccine protecting 50% of the

TABLE III. Demonstration of Immunological Type Specificity of Monovalent Foot-and-Mouth Disease Vaccines in Young Adult Mice.

Vaccine			Challenge virus				
				50% end-point*			
Type and batch	Log ID_{50} per ml	Dose, ml	Type	Vaccinated mice	Control mice	Protection afforded in log ID_{50}	Titer* of challenge virus in suckling mice
O V187E†		.4	O	<1.0	4.5	>3.5	7.6
			A	4.3	4.7	.4	7.5
			C	4.1	"	.6	6.6
A 3F‡	6.5	"	O	5.6	5.4	Nil	7.5
			A	2.1	5.1	3.0	7.8
			C	4.0	4.2	.2	6.4
A 8F		"	O	5.5	5.3	Nil	7.5
			A	2.6	5.6	3.0	8.0
			C	5.1	5.0	Nil	8.2
C 1F	6.8	.2	O	5.4	5.8	.4	7.5
			A	4.3	4.8	.5	7.6
			C	<1.0	5.1	>4.1	7.0
C 2F	6.6	"	O	5.6	5.6	Nil	7.3
			A	5.3	5.5	.2	7.2
			C	1.8	4.3	2.5	7.5

* Negative powers of 10.
† E—Tongue epithelium virus (Venezuelan vaccine).
‡ F—Frenkel culture virus.

vaccinated mice.

The results of testing vaccines in mice must also be correlated with the results of testing the same vaccines in cattle but the experience gained in the experiments described is sufficiently promising to suggest that further work may show that mice can be used as substitutes for cattle in testing the potency of foot-and-mouth disease vaccines.

Summary. Foot-and-mouth disease vaccines inoculated by the subcutaneous route in young adult mice conferred protection against challenge by inoculation of virus strains adapted to young adult mice. These mice were 3 to 6 weeks old weighing 9 to 13 g at time of vaccination.

Author thanks Dr. W. M. Henderson, Director of Center, for criticisms and review of text and Ephigenio Sobral for technical help.

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Response of Epididymal Adipose Tissue to Small Concentrations of Insulin: Effect of Cortisone.* (25641)

P. Riet Correa, E. Magalhaes and M. E. Krahl[†]
Inst. de Fisiologia Experimental, Faculdade de Medicina, Universidade do Rio Grande do Sul, Porto Alegre, Brazil

Addition of insulin to rat epididymal adipose tissue *in vitro* causes increases in glucose uptake(1-4), in CO_2 evolution with glucose as substrate(2,5,6,7), in fat synthesis(2), and in incorporation of amino acids into protein (8). The stimulatory actions upon glucose uptake and CO_2 evolution have been proposed as the basis for insulin detection methods(5, 6,9). In attempting to assay insulin quantitatively it has been found, in confirmation of Ball *et al.*(6), that the response to insulin of adipose tissue from successive rats of a given colony may undergo sudden changes which negate the assay procedure. The causes of these changes are being studied. The present experiments show that sensitivity of adipose tissue to small concentrations of insulin added *in vitro* is significantly decreased by injection of cortisone acetate into the donor rat from which the adipose tissue is taken.

Methods. Non-fasting[‡] Wistar rats weighing 124-168 g, were sacrificed by cervical separation attended by minimal previous excitement. The 2 epididymal fat pads were transferred to filter paper which had been well moistened with the medium to be used for incubation, *i.e.*, Krebs-Henseleit-bicarbonate solution containing 200 mg % each of glucose and gelatin(6). Each pad was quickly divided into 3 approximately equal pieces. Each piece was transferred to 1.9 ml of the above medium in the main compartment of a 15 ml Warburg flask; the pieces were added in a predetermined and known order. The flasks were equilibrated with 95% O_2 - 5% CO_2 and incubated 3 hours at 37°C at a shaking rate of 120 oscillations per minute and an amplitude of 6 cm; 0.1 ml insulin solution was tipped in from the side-arm at end of the first hour to give the final concentrations shown; after incubation, the fat pads were drained on filter paper and weighed. Before insulin addition, consumption of oxygen and evolution of CO_2 are usually such as to give only a small pressure change with time; after insulin, there is increased CO_2 evolution, presumably associated with increased fat synthesis(6). In Fig. 1, the gas exchange is arbitrarily recorded as μl CO_2 per g tissue, *i.e.*, mm pressure change x K_{CO_2}/tissue weight in g). In Table I, the results are expressed as net *increase* in CO_2 evolution after insulin (μl CO_2 per g tissue per hour); this is calculated by subtracting pressure change for the first hour (before insulin addition) from that for the third hour (period of maximal insulin response), multiplying by K_{CO_2}, and dividing by tissue weight. Values for glucose uptake were also measured (10) on the same samples and include uptake for the equilibration period of 15 minutes, plus one hour of incubation without insulin, plus 2 hours with insulin. Insulin was prepared by diluting Lilly U-80 insulin with the incubation medium. Identical responses were obtained in trials with Lilly crystalline insulin[§] stated to contain approximately 0.4% glucagon (Lot No. 535664).

Results. The changes in CO_2 evolution and glucose uptake in response to insulin were found to vary according to the position in the fat pad from which the fragment was removed, the left distal portion being the most responsive of the 6 pieces to insulin, just as it is the most responsive of 4 pieces cut from 2 fat pads(7). Accordingly, each concentration of insulin was always tested in duplicate upon

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[*] Aided by grants from Rockefeller Foundation and Conselho Nacional de Pesquisas do Brazil.

[†] Permanent address: Dept. of Physiology, Univ. of Chicago.

[‡] Rats were provided *ad lib.* with diet containing the following as % by weight: milk, 64; bread, 20; wheat, 8; corn, 8; dried beer yeast, .2; cod liver oil, .08, and alfalfa were added to the ration during one day weekly.

[§] Insulin was kindly supplied by Dr. W. R. Kirtley, Eli Lilly and Co., and cortisone acetate by Dr. Mauricio Teichholz, Schering, S. A.

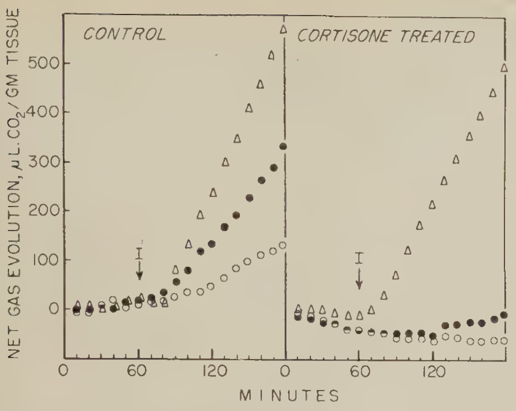

FIG. 1. Effect of prior injection of cortisone on response of rat epididymal adipose tissue to insulin added *in vitro*. Insulin concentrations, as microunits/ml, were: ○, 20; ●, 50; △, 10,000. Insulin was added at 60 min. For details, see *Methods*.

the same 2 segments of the fat pads from a single rat, *e.g.*, 20 μu/ml upon the right distal and left proximal portion, 50 μu upon the right and left middle portions, and 10^4 μu upon the right proximal and left distal portions; averages of the 2 duplicates are recorded. This procedure yields a log dose-response line with approximately the same slope as that reported for 4 pieces of fat from one rat(6).

Cortisone acetate,[§] suspended in 0.9% NaCl, was given subcutaneously, 2 mg per rat per day (1 mg each at 8 a.m. and 5 p.m.) for 3 days plus a final dose of 1 mg 3 hours before sacrifice. Control rats of nearly the same weight received the saline solution concurrently.

The effects of insulin and cortisone upon gas exchange are illustrated in Fig. 1, which presents the results for a single experiment, and summarized in Table I. Results for the controls are qualitatively similar to those for normal rats(6). Prior injection of cortisone acetate did not significantly change the slope of the curve for CO_2 evolution during the first hour, prior to addition of insulin; however, it significantly reduced *in vitro* stimulation of CO_2 production resulting from addition of 20 or 50 μu insulin per ml and had a similar but less significant antagonistic action toward 10^4 μu per ml (Table I).

The increases in glucose uptake produced by the same concentrations of insulin were also smaller with adipose tissue from the cortisone-injected rats than with tissue from saline injected rats; in fact, no stimulation above the control level was obtained with 20 or 50 μu insulin per ml. A single dose of 1 mg cortisone acetate 3 hours before tissue removal did not prevent insulin stimulation.

Discussion. The response to insulin of adipose tissue from successive rats of a given strain and colony may spontaneously decrease, with no significant change in basal glucose uptake or net gas exchange without insulin. Or, basal glucose uptake and CO_2 evolution may spontaneously increase to such high values that addition of small doses of insulin *in vitro* has no further effect(6‖). The present experiments show that the first of these anomalies can be reproduced by injection of cortisone acetate, suggesting that the spontaneous decrease in sensitivity may stem in part from excess endogenous adrenal steroids.

The relation of cortisone to the effects of very small concentrations of insulin does not appear to have been previously studied in individual tissue. There is evidence that insulin in sufficiently high concentrations can partly or wholly counteract the inhibitory effects of

TABLE I. Effect of Cortisone Acetate on Glucose Uptake and CO_2 Production of Epididymal Adipose Tissue in Presence of Small Concentrations of Insulin. Cortisone acetate injected into donor rats as described in text; insulin added *in vitro*.

Insulin conc., μunits/ml	Adipose tissue from control rats	Adipose tissue from cortisone-inj. rats	Significance of difference, P, between control and cortisone samples
Net increase, after insulin, in gas evolution, calculated as mm³ (μl) CO_2/g/hr			
20	80 ± 6.6*	22 ± 2.2	.001
50	125 ± 8.9	41 ± 4.7	.001
10,000	298 ±26.	220 ±23.	.04
Glucose uptake, mg/g/3 hr			
0	1.4 ±.21	1.4 ±.15	
20	1.9 ±.19	1.2 ±.29	.06
50	2.9 ±.33	1.1 ±.20	.001
10,000	4.9 ±.32	2.9 ±.50	.005

* Mean ± stand. error; means are obtained from duplicate tissue samples from each of 8 rats, except for the values for glucose uptake without added insulin; each of these is mean of 9 duplicate samples from 3 rats.

‖ Unpublished experiments.

cortical steroids on CO$_2$ evolution or fatty acid synthesis by mammary gland slices(11), on fatty acid synthesis by rat adipose tissue (12), or on glucose uptake by rat diaphragm (13,14,15).

Summary. The stimulation of glucose uptake or CO$_2$ evolution produced by 20, 50, or 10^4 μu insulin per ml *in vitro* to rat epididymal adipose tissue was markedly reduced by prior subcutaneous injection of the rat tissue donor with 7 mg cortisone acetate in divided doses over a period of 3½ days.

The authors are indebted to Prof. Oscar Homrich for the loan of the Warburg apparatus.

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Action of Mucoproteins *in vivo* and in Tissue Culture on Poliomyelitis Virus. (25642)

N. ERCOLI, G. BOBALIK, R. K. STUBBS AND O. NUNEZ-MONTIEL
Dept. of Pharmacology and Chemotherapy, Armour Pharmaceutical Co., Kankakee, Ill., and Inst. Venezolano de Investigaciones Cientificas, Caracas, Venezuela

The present report contains our findings on the protective effect of various mucoprotein fractions obtained from hog gastric mucosa in experimental poliomyelitis infection of mice (Lansing strain II) and the influence of the same proteins on development of the virus in monkey kidney cells.

Materials and methods. Three hog gastric mucosa fractions (A,B,C) received from Prof. V. Capraro (Milano, Italy) and 2 extracts (D,E) prepared in the Armour Laboratories by E. Samsa, V. Snider and P. Van Melle were used for study. The characteristics of these substances as determined by V. Capraro are: (A) A and H group specific substance, 95% pure by electrophoretic determination (free phase). Anti-agglutinating potency 10^7. (B) "B$_{12}$ binding factor"(1) 100% pure. B$_{12}$ binding titer, 22 μg/mg in the *E. coli* assay. (C) Acid aminopolysaccharide. Electrophoretically 95% pure, uronic acid content 35%. (D) Armour preparation KO-45-102-1. Uronic acid content 1.62%. (E) Armour preparation KO-45-124. B$_{12}$ binding titer 228 mμg/mg, uronic acid content 1.6%. Doses of 1 and 2 μg showed A, respectively H substance activity, inhibiting 10 minimal agglutinating doses in the systems A red cells/anti A serum and O cells/anguilla anti H serum.

Infection. Swiss mice of 14-18 g, 4-5 weeks of age, were inoculated intracerebrally with 0.03 ml Lansing strain (Type II) virus, diluted 1:3000. Standard procedure was to infect mice Friday and begin treatment Monday; however on 4 groups of mice treatment was started prior to inoculation. Ten treatments were given, 2 daily for a period of 5 days, except when otherwise stated. Experimental animals were observed daily for a pe-

TABLE I. Action of Mucoproteins in Experimental Poliomyelitis (Lansing II) Infection of Mice.

Exp. No.	Preparation	Dose, mg/kg × No. treatments	Route	Survivors/total mice after:			
				10 days	20 days	30 days	60 days
I	A	10 × 10	Subcut.	10/10	7/10	4/10	3/10
		2.5 ”		”	9/10	6/10	”
	B	10 ”		6/10	4/10	2/10	2/10
		2.5 ”		9/10	7/10	3/10	3/10
	C	10 ”		”	4/10	2/10	2/10
		2.5 ”		”	”	”	”
	E	10 ”		10/10	6/10	5/10	4/10
		2.5 ”		9/10	”	4/10	3/10
		Controls		”	4/10	0/10	
II	E	20 × 10	Oral	10/10	2/10	1/10	1/10
		Controls		”	”	0/10	
III	D	20 × 10	Oral	14/15	8/15	8/15	7/15
		10 ”		13/15	5/15	2/15	2/15
	E	20 ”		12/15	11/15	9/15	8/15
	γ-globulin	20 ”		”	”	7/15	2/15
		Controls		24/30	7/30	3/30	1/30
IV	E	40 × 2	Oral*	9/10	4/10	2/10	2/10
		40 × 1	*	”	5/10	1/10	1/10
		20 × 2	Subcut.*	8/10	4/10	”	”
		10 ”	*	10/10	8/10	5/10	5/10
		20 × 10		8/10	6/10	4/10	3/10
		10 ”		10/10	8/10	”	”
		2.5 ”		”	7/10	5/10	4/10
		Controls		7/10	4/10	0/10	

* Treatment started prior to inoculation.

riod of 60 days. Death followed appearance of paralysis by 3 to 6 days. For experiments on isolated cells, monkey kidney (*Macacca mulatta*) was prepared according to Bodian's technic(2) and suspended in Hank's solution. The medium was changed to Earle's solution when the cells showed a completed sheet (after 5-6 days). The latter solution was used for dilution of the mucoprotein also. Virus growth has been estimated by cytopathological observation. Moreover, the infectivity of the test tube aliquot, after completed cell infection, was measured by transfer to new tissue cultures.

Results. The therapeutic effects observed *in vivo* are presented in Table I. It would seem that there is no dose-effect relationship in the ranges studied and no significant difference between repeated oral and subcutaneous administration of the mucoprotein. Therefore, and in view of the differences among experiments, it is justifiable to evaluate the results in their totality: by adding survival rate at the 30 day observation period (as generally done), administration of 10 doses of preparation E gave 42.5% protection (32 out of 75), of preparation D, 33.3% (10 out of 30), of preparation A (blood group substance) 50% (10 out of 20). Considering all animals treated, regardless of preparation and dose, and including groups treated once or twice only, survival rate amounts to 33.5% (70 out of 205) compared to 5% in the untreated groups (3 out of 60). The difference between total survivors of treated groups in comparison to controls was just as marked at the 60 day observation period: 28.2% (59/205) in treated and 1.7% (1/60) in control groups. In one experiment, 20 mg/kg gammaglobulin administered orally 10 times resulted in a protective effect of the same order as with mucoprotein.

In tissue cultures, toxicity of the mucoprotein (preparation E) for monkey kidney cell was first determined: 200 μg/tube induced slight changes, while no effect was noticed with lower (2-100 μg/tube) doses after 4 days of growth. In the tubes used for toxicity experiments (10-200 μg mucoprotein), inoculated the fifth day with 100 TCD_{50}

(10^{-4}) virus, growth seemed to be slighter than in the controls 2 days after inoculation. However, one day later, virus growth in all 45 tubes was the same as in the controls. In a second experiment, to control more closely the possible retarding effect of mucoprotein on the infection, virus inocula decreasing to the limit of infectiveness (from 10^{-4} to 10^{-8}) were used, with a fixed dose, 100 μg/tube, of mucoprotein; in one series of tubes the mucoprotein was kept in contact with the cells for 24 hours, then washed (before inoculation), in another, it was present for the duration of the experiment. The mucoprotein induced no measurable effect on virus development and infectivity in this experiment including 2 series of 5 replicate tubes for each of the 5 virus dilutions used. In another experiment of the same size, previous exposure of the cells for 4 days to 100 μg mucoprotein or even its continued presence after inoculation did not influence growth and infectivity. Nor did mixing 10^{-4} to 10^{-8} inocula with 100 μg mucoprotein for one hour at room temperature change virus growth. In neutralization test, using mice for test of virulence, up to 2500 μg/ml mucoprotein had no direct effect on the virus: all 50 mice inoculated died at the same time as the controls. (In the same test, the minimal neutralizing dose of gammaglobulin, 625 μg/ml, gave 100% survival.)

Conclusions. It appears that certain mucoproteins exert a protective action against experimental poliomyelitis infection of mice and, interestingly, by oral administration also. This action does not seem to be related to virus neutralization, nor to an interference of virus uptake by the cell, or to virus multiplication and infectiveness, according to the results of tissue culture experiments. Excluding the direct action of mucoprotein on the virus, on its cellular uptake and multiplication, the hypothesis of a "nonspecific" effect, whereby treatment would increase in some manner the nonspecific resistance of the organism, becomes suggestive. The lack of dose-effect relationship is favorable to such an hypothesis, though it is quite possible that the dose range was above the minimal required to elicit protective action. The acid mucoprotein (C) and the B_{12} binding substance (B) appeared to be less effective than the blood group substance (A) in the single experiment in which they were compared. However, our data are not sufficient for quantitative comparison between different types of preparations. Whether the action noted is an integral property of this and perhaps other types of mucoproteins, or related to a single, more simple component or subunit, such as a mucopolysaccharide, requires further investigation.

Summary. Mucoproteins obtained from hog gastric mucosa exert a protective effect in experimental poliomyelitis (Type II) infection of mice, while they have no action on the virus in tissue cultures. The protective action is attributed to some mechanism increasing nonspecific resistance of the host.

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Transfer of Radioiodinated Human Serum Albumin (RISA®) from Cerebrospinal Fluid to Blood Plasma.* (25643)

C. A. VAN WART, J. R. DUPONT, L. KRAINTZ (Introduced by H. E. Hoff)
Department of Physiology, Baylor University College of Medicine, Houston, Texas

There have been many conflicting reports regarding the site of reabsorption of cerebrospinal fluid. Cerebrospinal fluid protein plays a significant role in association with many pathological states of the CNS; however, little is known about the dynamics involved. Behring(1), Sweet(2), and others(3) have described transfer of various ions and non-

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electrolytes between blood stream and cerebrospinal fluid. Apparently each plasma constituent has its own rate of exchange. Until quite recently, nearly all work on CSF dynamics had been done with dyes and colloids, or particulate matter only(4). Use of radioactive tagged serum albumin offered a promising approach to the problem of protein transfer between CSF and plasma. Fishman (5) obtained an equilibrium time on dogs of 18-20 hours for albumin by administering radioiodinated serum albumin and monitoring cisternal fluid and plasma. Sweet's data with 2 patients gave quite varied rates of clearance from the CSF for intrathecally administered radio-albumin.

Our purpose was to examine plasma to determine appearance time and equilibrium time for intracisternal administered radioiodinated serum albumin in dogs. Concentration of CSF-protein is somewhat lower in the dog than in the human, but all other considerations indicate that the dog is an ideal test animal for this purpose. A control study was also done to demonstrate whether colloidal gold would pass from CSF to plasma under similar conditions.

Methods. The dogs were anesthetized with a combination of morphine sulfate and sodium pentobarbital. Doses of 25 μc of RISA (Abbott) containing approximately 0.5 mg of albumin were prepared in ½ ml volume after passing it through an anion exchange resin to eliminate ionic iodide. A series of 12 mongrel dogs averaging 13.5 kg received ½ ml of the above preparation into the cisterna magna *via* a 3 inch 22 gauge spinal needle. Blood samples were taken at varying time intervals up to 24 hours from the whole blood. Radioactivity was assayed in a well type scintillation gamma counter. All samples were counted for at least 5000 counts and corrected for background. The nature of the plasma radioactivity was determined by scanning paper electrophoresis strips of the samples. To compare data obtained from different animals, radioactivity of the plasma was calculated as percent of equilibrium. One hundred percent equilibrium was defined as amount of radioactivity which would have been present in the total circulating plasma had the radioactivity been administered intravenously. Circulating plasma volumes of the dogs were determined by the dye dilution technic using Evans Blue. In addition, 3 dogs were given ½ ml of Au-198 (Aurcoloid, Abbott) intrathecally. A clear tap was demonstrated in all instances before injection. These dogs were scanned for radioactivity utilizing the same technics as for thyroid gammagrams. The blood of these dogs was also radioassayed.

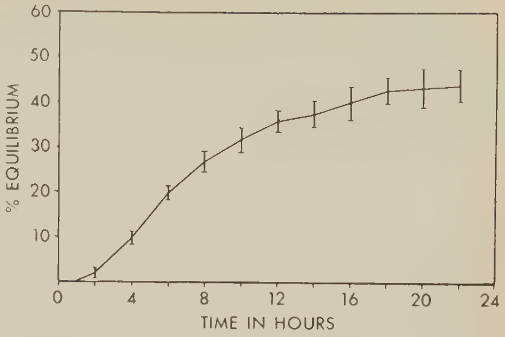

FIG. 1. Appearance of radioactivity in plasma after intrathecal instillation of RISA. One hundred percent equilibrium is defined as level of plasma radioactivity which would be found if total inj. dose was completely transferred from CSF to plasma. Values at each time interval represent mean ± stand. error obtained in 12 dogs.

Results. Fig. 1 shows the appearance of radioactivity in plasma after intrathecal instillation. The curve represents the average of 12 dogs. After intrathecal injection, RISA migrates to the plasma. Little radioactivity was found in blood within the first hour, however levels significantly above background appeared at the end of 2 hours. Sensitivity of the determinations at that point was limited by amount of radioactivity administered, plasma volume, time needed for mixing of the injection within the CSF-space, and sensitivity of the counting equipment. There was a fairly rapid increase over the first 16-18 hours, when the curve levels off. Constant values were found between 16-20 hours in these experiments. At equilibrium there was no further increase in radioactivity of the plasma. Paper strip electrophoresis of the plasma samples showed that radioactivity was associated with the albumin fraction.

When Au-198 was injected intrathecally and the blood radioassayed for its appearance, no values above background could be detected for a 24 hour period. A gammagram at 26 hours indicates that the colloidal gold was localized within the cranial cavity of the dog. Most of the gold appears in the midline with only a slight extension down into the spinal space. This lack of extensive migration within the fluid space is perhaps due to the horizontal position of the dog, or rapid phagocytosis of colloid by CSF-macrophages. No radioactivity was detected in the region of cervical or thoracic lymph-nodes. The spleen and liver were also not above background.

Discussion. The methods used offer several advantages. Repeated taps of a small space such as the CSF are avoided, and trauma or leakage around the puncture-needle brought to a minimum. No special care need be taken in collecting the sample or regarding the position of the dogs during experiment. Negligible amounts of protein are injected so as not to upset concentration or dynamics of the fluid.

In the present study the value of 16-20 hours agrees well with the equilibrium time reported by Fishman. Data obtained by Sears *et al.*(6) indicate, however, that over 24 hours approximately 52% of intravenously injected RISA is lost from the plasma; only a fraction of this, in all likelihood, going to the CSF. Similar results were obtained under our experimental conditions. The radio-albumin entering the blood stream from the cerebrospinal fluid is hence distributed in the same manner. After RISA begins to leave the CSF-space it is distributed throughout plasma, lymph, interstitial fluid, and presumably the CSF. The failure of the curve in Fig. 1 to reach more than 45% of the calculated equilibrium is thus clearly explained. The 3-5% remaining is ascribed to experimental error and the rest left in the cerebrospinal fluid.

Under the condition of our experiments Au-198 was not detected outside the CSF. Colloidal dyes have been reported to collect around the arachnoid villi, in the perivascular spaces, or around nerve roots. Wollard(7) noticed that mesothelial cells ingested Trypan blue. This behavior of colloidal particles was taken as evidence that the Pachionean bodies were major factors in CSF absorption. Although the present study cannot confirm this, it does indicate that lymphatic drainage or a route involving the Virchow-Robin's spaces are not of importance in absorption of colloids of the size of colloidal gold. Added support is thus lent to Bowsher's(8) leptomeningovascular pathway.

Summary. Passage of albumin from cerebrospinal fluid to blood plasma was determined in anesthetized dogs by injecting radio-iodinated human serum albumin into the cisterna magna and monitoring the plasma radioactivity. An equilibrium time of 16-20 hours was obtained. Radioactive colloidal gold injected intrathecally was not detectable in the general circulation in 24 hours and appeared to be well localized within the cerebral fluid space.

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Effects of Chlorothiazide on Plasma Electrolytes in the Nephrectomized Dog. (25644)

W. R. Beavers

Dept. of Pharmacology, University of Texas Southwestern Medical School, Dallas

Chlorothiazide and structurally related compounds, orally effective diuretics, are widely used as antihypertensive agents. It has been suggested that chlorothiazide lowers blood pressure by means other than sodium excretion(1), but a definite mechanism has not been demonstrated. It was considered therefore, to be of interest to evaluate the effects of chlorothiazide on plasma electrolytes in the nephrectomized animal.

Methods. Fifty adult mongrel dogs were anesthetized with sodium pentobarbital (35 mg/kg) and arterial blood pressures were measured with a mercury manometer recording on a smoked drum. Acute bilateral nephrectomies were performed on all dogs from a ventral approach. Blood samples were taken prior to nephrectomy immediately prior to administration of experimental drugs, and 1, 1½ and 2 hours following beginning of drug injection. Blood hematocrits were determined, and plasma was analyzed for potassium and sodium with a Beckman flame photometer, and for pH with a Beckman pH meter. A control group of 8 animals received 10 ml 5% glucose in water one hour after nephrectomy followed by a 30 minute infusion of 50 ml 5% glucose. All experimental animals received a like amount of 5% glucose, and the second group of 9 animals had in addition 20 mg/kg chlorothiazide (Diuril) initially and in the 30 minute infusion. The third group of 13 animals were given 2.5 mg/kg mercury in the form of mercaptomerin (Thiomerin) as a prime dose and a like amount during the 30 minute infusion. The fourth group of 10 animals received 2 doses of 2 mg/kg hydrochlorothiazide (Hydrodiuril)* given in a like manner, and the last group of 10 animals similarly received a prime dose of 10 mg/kg acetazolamide (Diamox) and a 30 minute infusion of 10 mg/kg acetazolamide.

Results. Table I summarized effects observed on plasma potassium and sodium concentrations of all drugs tested. There was no change in plasma sodium induced by any of the compounds. Plasma potassium values during the test period were unaltered in control animals, and in animals which received mercury or acetazolamide. However, in animals receiving chlorothiazide and hydrochlorothiazide there is a significant decrease in plasma potassium one hour after drug administration was started. This lowered potassium level continued for at least 2 hours after drug administration.

Fig. 1 presents graphically the change in plasma potassium seen in the 5 groups. At the 1½ and 2 hour post-treatment periods, potassium concentrations were decreased approximately 15% in both chlorothiazide and hydrochlorothiazide treated animals. Statistical treatment of the change in potassium values reveals that in these groups a significant ($P = <0.05$) reduction occurred when compared to changes seen in other groups.

There were no other significant differences between groups in the parameters studied. Mean arterial blood pressures of all animals averaged 126 mm Hg after nephrectomy and a mean decrease of 18 mm pressure was found after 2 hours. Decreases were similar in all groups. Hematocrits rose an average of 3% during the test period, with no intergroup differences found. Venous blood pH did not vary significantly in any group.

Discussion. A previous study from this laboratory(2) indicated that chlorothiazide decreases vascular reactivity in the anesthetized dog. The effect was not unique with chlorothiazide, being seen also with acetazolamide, dichlorphenamide and mercaptomerin (3).

The present results strongly suggest that chlorothiazide and hydrochlorothiazide have some effect on potassium distribution in the nephrectomized dog, an effect not shared by

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* Diuril and Hydrodiuril supplied by Merck, Sharp and Dohme, Inc.

TABLE I. Plasma Electrolyte Values in Nephrectomized Animals.

Group & No. animals	Control	Time in hr after drug 1	1½	2
		K, meq/l		
Control 8	3.70 ± .23†	3.52 ± .21	3.50 ± .24	3.60 ± .18
Chlorothiazide 9	3.98 ± .15	3.50 ± .05*	3.32 ± .14*	3.25 ± .17*
Mercury 13	3.75 ± .17	3.71 ± .20	3.67 ± .19	3.78 ± .20
Hydrochlorothiazide 10	3.74 ± .11	3.30 ± .13*	3.21 ± .11*	3.31 ± .11*
Acetazolamide 10	3.70 ± .19	3.74 ± .17	3.70 ± .16	3.75 ± .19
		Na, meq/l		
Control 8	141.4 ± 1.8	140.91 ± 2.1	141.94 ± 1.8	140.47 ± 1.9
Chlorothiazide 9	142.4 ± 2.5	143.61 ± 2.8	145.07 ± 2.6	142.54 ± 3.9
Mercury 13	147.7 ± 1.6	148.3 ± 2.8	147.7 ± 1.4	147.8 ± 1.1
Hydrochlorothiazide 10	148.0 ± 1.2	147.39 ± 1.2	146.76 ± 1.8	149.81 ± 1.2
Acetazolamide 10	148.9 ± 1.4	149.4 ± 1.5	148.5 ± 1.5	149.1 ± 1.6

* Significantly different from control values, P = <0.01. † ± stand. error.

mercaptomerin or acetazolamide. Whether this extrarenal effect is pertinent to observations that chlorothiazide reduces blood pressure in human hypertensives without necessarily altering body sodium, as reported by Winer(4), must be determined by future studies. These effects on potassium levels are observed using high doses of chlorothiazide and hydrochlorothiazide, and thus an easy extrapolation to the clinical situation is not possible. Acetazolamide, a potent carbonic anhydrase inhibitor, had no observable effect on plasma potassium, therefore the known carbonic anhydrase inhibition produced by high doses of chlorothiazide(5) is not considered responsible for the potassium changes.

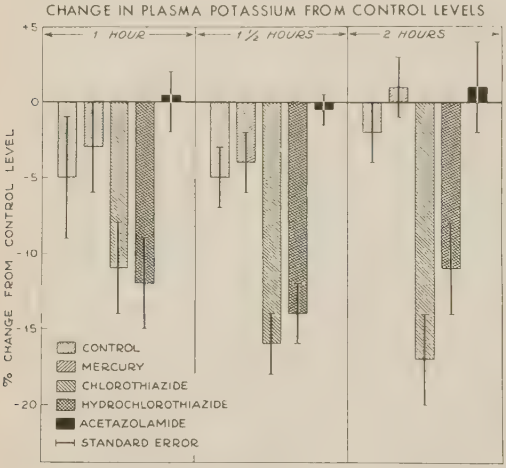

FIG. 1.

Summary. Chlorothiazide and hydrochlorothiazide significantly lowered plasma potassium levels in the nephrectomized dog after intravenous administration. Mercaptomerin and acetazolamide had had no such effect. Plasma sodium concentrations were not altered and no differential effects on pH, hematocrit, or blood pressure were seen in any groups of animals. These findings suggest that chlorothiazide and hydrochlorothiazide have an extrarenal effect not shared by mercaptomerin or acetazolamide.

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Pulmonary Emphysema and Polycythemia Induced in Rats by Forced Swimming.* (25645)

Sante Tura† (Introduced by John H. Lawrence)
Donner Lab. and Lawrence Radiation Lab., University of California, Berkeley

In chronic pulmonary emphysema there is an inadequate response of erythropoietic activity to low arterial oxygen saturation(1-10). The following experiments show that chronic pulmonary emphysema induced in rats by forced swimming is associated with polycythemia.

Materials and methods. Seventy-three adult male rats of Long-Evans strain weighing between 250 and 325 g were used. Sixty were placed daily in 2 pools (3 x 3 x 2 feet) with water temperature of 22°C. Every day 10 at a time were made to swim to exhaustion. Exhaustion was produced in 15 minutes during the first month, after which the rats could not swim more than 10 minutes. The experiment was continued 90 days when only 29 rats were alive; death of the others was due to drowning. In surviving rats and controls the following studies were performed. 1) *Roentgenogram of chest.* Under deep ether anesthesia, which resulted in marked reduction of respiratory ventilation, one experimental and one control rat were placed over the same film. The roentgenograms prepared for gross and densitometric study were made on Eastman Industrial x-ray film type AA. Exposures were made at target-to-film distance of 1 meter, with approximately 2 seconds at 68 kilovolts, 10 milliamperes. Development was in Eastman Standard X-ray developer 5 minutes at 20°C (Fig. 1). 2) *Densitometry.* Roentgenograms of chest of both experimental and control animals were scanned for differences in light transmission by a spectographic microdensitometer‡ at constant speed of 1 mm/min. The light slit was 100 μ by 0.1 mm. Three parallel paths of scanning were used of lengths between 2.5 and 3.0 cm; the separation between paths was 0.4 cm. The direction of scanning was from caudal to cephalad. Light transmission values were recorded by Leeds and Northrop Speedmax recorder. Before and after scanning of each roentgenogram, both zero and sensitivity settings of the densitometer were controlled. By end of complete scanning operation the drift was less than 1%. 3) *Hematological determinations.* Blood volume was determined by Fe^{59} tagged cell technic. Labeled blood was drawn into a heparinized syringe by cardiac puncture from a donor that had received 10 μc of Fe^{59} intraperitoneally one week before. Blood volume determination was done under ether anesthesia. After injection of 0.5 cc of donor blood through the saphenous vein, 6 minutes were allowed for mixing after which 2 cc of blood were drawn from jugular vein. An aliquot of this blood was used for counting and for hematocrit and hemoglobin determinations. Amount of radioactivity injected was divided by amount of radioactivity/unit of recipient blood to give the assumed blood volume. This, multiplied by the hematocrit, gave total red cell volume. To be more comparable, blood, plasma and red cell volumes were expressed in cc/100 g of body weight. The hematocrit was determined in Wintrobe's tubes, hemoglobin by method of Turner(11). 4) *Histological sections.* Lungs were fixed by injection of 10% formalin intratracheally before opening the chest. Adrenals were carefully dissected under binocular microscope and weighed. Hematoxylin-Eosin stain was used for both lung and adrenal tissues. For lung tissue Gomori's Aldehyde-Fuchsin stain for elastic fiber and Perl's Prussian blue were used.

Results. Determinations were made of percent light transmission by scanning of chest films. Three determinations were made in each intercostal space of right lung from second to eighth rib, and the results compared with those of controls scanned in topographi-

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† Donner Postdoctoral Fellow and Fulbright grantee; at present Medical Assistant, Inst. of Medical Pathology, University of Bologna, Italy.

‡ Janel Ash Co.

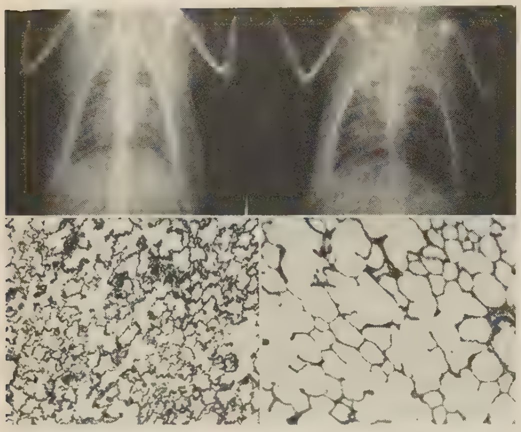


FIG. 1. Chest roentgenogram of control (left) and experimental (right) rats. In the experimental, decrease of density is accompanied by increase of diameter of the rib case. Microphotographs of lungs of same rats are shown below.

cally correspondent places. From Fig. 2 it is evident that the difference in density ranges from a minimum of 15% in seventh intercostal space to a maximum of 24% in fourth intercostal space. The decrease in density obtained from negative films means an increase in amount of air in pulmonary alveoli. This increase is an essential sign for radiological diagnosis of pulmonary emphysema. Total red cell volume was increased 24% in rats that swam (from 2.1 ± 0.17 cc/100 g to 2.6 ± 0.22 cc/100 g ($P<0.001$). No change in hematocrit or hemoglobin was observed. Adrenal weight of rats made to swim averaged 44 ± 1.1 mg compared with 37 ± 1.8 mg for controls ($P<0.01$). Microscopic examination of lungs of experimental group revealed emphysema which was mild in most cases but marked in a few (Fig. 3), and was characterized by dissolution of alveolar walls with formation of lacunae. Generally alveolar lumens were larger than normal. The elastic fiber stain showed a disruption of fibers and the iron stain showed presence of old hemorrhage.

There was direct correspondence between values from densitometry of chest films and histological changes. The adrenal glands of both groups were histologically similar.

Discussion. Pulmonary emphysema as demonstrated both by roentgenogram of the chest and histological examination of lungs was produced in rats by swimming to exhaustion daily for 90 days. These same rats had a consistent mild polycythemia with average 24% increase in total red cell volume.

It is known(12) that blood volume/g body weight decreases with age in rats (from 2.2 cc/100 g of red cell volume 60-150 days of age to 2.0 cc/100 g after 200 days of age) because the accumulated fat is less well-vascularized than lean tissue. It was possible that exercise prevented deposition of fat and consequently prevented physiological decrease in blood volume expected with age. Body density of control and experimental rats was measured by the method of Siri(13) and the same average values were found for both groups. Since swimming to exhaustion in cold water should provide sufficient stress to stimulate the adrenals(14) and since it is known that increased adrenal activity may produce an increase in circulating erythrocytes(15,16, 17), the adrenals were carefully dissected and weighed. A correlation between increase in adrenal weight and increase in total circulating red cell volume was found (Fig. 4).

The stress of swimming to exhaustion resulted in pulmonary emphysema, increase in total circulating red cell volume and adrenal hypertrophy. The mild polycythemia could be secondary to both increased adrenal activ-

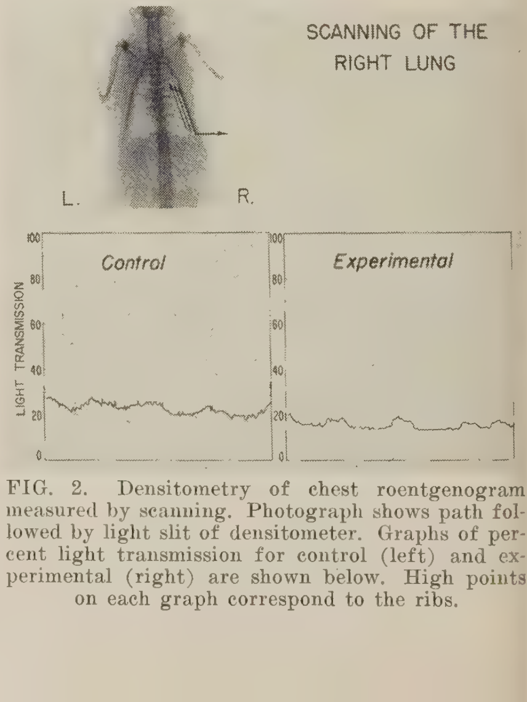
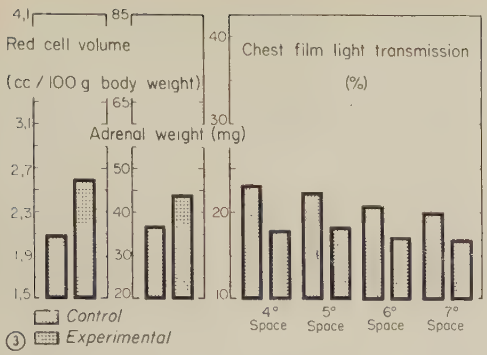


FIG. 2. Densitometry of chest roentgenogram measured by scanning. Photograph shows path followed by light slit of densitometer. Graphs of percent light transmission for control (left) and experimental (right) are shown below. High points on each graph correspond to the ribs.



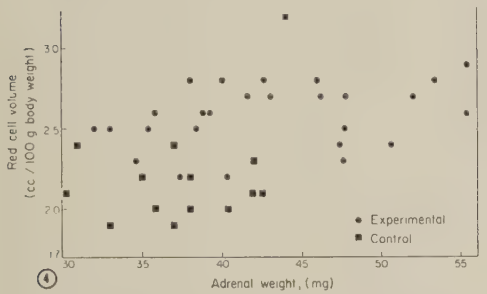


FIG. 3. Red cell vol/100 g B.W., adrenal and % of light transmission obtained by scanning of chest roentgenograms, are compared for both control and experimental groups.

FIG. 4. Shows correlation between red cell vol and adrenal wt.

ity and hypoxia due to pulmonary emphysema. After treatment with ACTH Garcia *et al.*(15) observed an increase of 30% in red cell volume accompanied by 100% increase in adrenal weight in the same strain of rats used in these studies. In the present experiment there was 24% increase in total circulating red cell volume with a relatively small increase in adrenal weight (18%).

This suggests that the degree of adrenal hypertrophy was insufficient to contribute markedly to polycythemia and that the emphysema must have been the primary factor responsible for increase in total circulating red cell volume. This finding is in contrast to the common clinical picture where emphysema is frequently not accompanied by polycythemia.

Summary. Chronic pulmonary emphysema was produced in rats by forcing them to swim to exhaustion each day for 90 days. The emphysematous changes in lungs were accompanied by increase in total circulating red cell volume and adrenal hypertrophy. The mild polycythemia could be secondary both to increased adrenocortical steroid output and hypoxia due to pulmonary emphysema.

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Occurrence of Ethylvanillic Acid in Human Urine and its Metabolism.* (25646)

Chris Nolan and Marvin D. Armstrong†
Lab. of Hereditary and Metabolic Disorders and Dept. of Biological Chemistry, University of Utah College of Medicine, Salt Lake City

Many laboratories are applying technics of paper chromatography to study of compounds in body fluids of patients with various diseases in the hope that new abnormalities of metabolism may be found. A pointed example of the caution which must be exercised in interpreting results of an exploratory chromatographic screening procedure is provided here by the finding that a phenolic acid which had been observed only in urine of severely mentally retarded institutionalized children is not only not of endogenous origin but is not even a naturally occurring substance. The phenolic acid, compound 28(1), was observed in urine of a few severely mentally retarded patients. It was excreted consistently by these, but not by other patients nor by normal persons. Much later, in connection with another problem, the metabolites excreted after ingestion of vanilla flavoring included a material which had the same chromatographic properties as compound 28. These vanilla preparations contained ethylvanillin (3-ethoxy-4-hydroxybenzaldehyde) in addition to vanillin, so ethylvanillic acid (3-ethoxy-4-hydroxybenzoic acid) was prepared; it has the same chromatographic properties as compound 28. The vanilla flavoring used in the kitchen of the institution was a solution of pure ethylvanillin in propylene glycol. The very severely retarded patients, among whom were those who excreted ethylvanillic acid, were maintained on semi-solid diets, which included considerable amounts of milk preparations and puddings, all highly flavored with vanilla. Thus, only a group of severely retarded patients were given enough synthetic flavoring agent to make it a prominent urinary metabolite. After ingestion of artificial vanilla flavoring or ethylvanillic acid another new phenolic acid, in addition to ethylvanillic acid, was found in urine. This material had properties identical with those of synthetic ethylvanilloylglycine. In addition to these 2 substances, a glucuronide of ethylvanillic acid was present.

Methods. Preparation of compounds. 3-Ethoxy-4-acetoxybenzoic acid (m.p. 154-155‡, Lit. 152-153°(2)) and 3-ethoxy-4-hydroxybenzoic acid (ethylvanillic acid) (m.p. 165°, Lit. 164-165°(2)) were prepared as described by King(2). 4.5 g of 3-ethoxy-4-acetoxybenzoic acid and 2.8 g of glycine ethyl ester hydrochloride were coupled by the method of Vaughn and Eichler(3). The resulting crude 3-ethoxy-4-acetoxybenzoylglycine ester was not purified but was saponified without further treatment. The 1.9 g (40% theor.) of crude 3 - ethoxy - 4 - hydroxybenzoylglycine (ethylvanilloylglycine) was recrystallized from 30 ml of hot water to yield 1.7 g of product; white needles, m.p. 161-162°. For analysis the product was again recrystallized from water: m.p. 162-163°. Analysis§ Calculated: for $C_{11}H_{13}O_5N$: C, 55.23%; H, 5.48%; N, 5.85%. Found: C, 54.81%; H, 5.64%; N, 5.71%. For routine screening, urine samples were extracted and the extracts subjected to 2-dimensional paper chromatography as described previously(1). Chromatographic properties of ethylvanillic acid and ethylvanilloylglycine are presented in Table I; properties of vanillic acid are included for comparison. For chromatographic identification of ethylvanillic acid and ethylvanilloylglycine, aliquots of solutions of the authentic compounds were added to extracts of control urine which contained neither, and chromatography was carried out in usual 2-dimension solvent pairs. Location of com-

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† Present address: Fels Research Inst., Yellow Springs, O.

‡ Melting points were taken in open capillary tubes and are uncorrected.

§ Analyses made by Weiler and Strauss Microanalytical Lab, Oxford, England.

TABLE I. Chromatographic Properties of Ethylvanillic Acid and Ethylvanilloylglycine.

	R_F				Color reactions		
Compound	Ipr-NH_3	Bz-Prop. acid	aqu. KCl	Bu-Ac. acid	Dz SA	Dz PNA	PDAB/Ac_2O
Vanillic acid	.20	.70	.64	.89	Orange	Purple	
Ethylvanillic acid	.32	.84	.64	.93	"	"	
Ethylvanilloylglycine	.23	.28	.71	.83	Pink	"	Greenish-yellow

Solvent systems: Ipr-NH_3, isopropyl alcohol - aqu. ammonia - H_2O, 8:1:1; Bz-Prop. acid, benzene - propionic acid - H_2O, 100:70:5; aqu. KCl, 20% aqu. KCl; Bu-Ac. acid, *n*-butanol - acetic acid - H_2O, 8:2:2.

Reagents: Dz SA, diazotized sulfanilic acid(7); Dz PNA, diazotized *p*-nitroaniline(8); PDAB/Ac_2O, *p*-dimethylaminobenzaldehyde in acetic anhydride(9).

pounds as well as color reactions described in Table I were compared with those on chromatograms prepared from extracts of urine obtained from patients and after ingestion of ethylvanillic acid and artificial vanilla flavoring, respectively. For quantitative estimation of free and conjugated ethylvanillic acid and ethylvanilloylglycine, urine samples were processed as described for determination of dopa metabolites(4). Suitable aliquots of ethyl acetate extracts of urine were applied to paper along with appropriately located graded amounts of authentic compounds. The chromatograms, containing both extract and standard compounds, were developed with usual solvent pairs and sprayed with diazotized *p*-nitroaniline; amount of compound in the extract was estimated by visual comparison of the spot produced by the unknown with the standards. The accuracy of this procedure was at least ± 15%. Completeness of recovery with the extraction procedure was tested by adding known amounts of authentic compounds to urine and determining recovery, which was quantitative for ethylvanillic acid and 85% for ethylvanilloylglycine. The values listed in Table II are corrected for incomplete extraction of ethylvanilloylglycine.

Results. Metabolic exp. Intake of tea, coffee, fruits, vegetables, and flavored beverages was restricted to minimize interference by phenolic acids of dietary origin. 100 mg and 500 mg portions of ethylvanillic acid dissolved in bicarbonate solution were taken by an adult male. Urine was collected for first 8 hours after compound was ingested, then for a second 8 hour period. Examination of second 8 hour collection showed that it contained less than 2% of ethylvanillic acid taken; values reported are only for the first 8 hour period. Amount of free ethylvanillic acid, ethylvanillic acid released by acid hydrolysis, and ethylvanilloylglycine excreted are listed in Table II. The acid labile conjugate was a glucuronide, since amount of ethylvanillic acid released by hydrolysis with β-glucuronidase (Ketodase, Warner-Chilcott) corresponded closely with that released by acid hydrolysis.

Recently, Hill, Ratcliffe, and Smith(5) commented upon the occurrence of ethylvanillic acid along with vanillic acid in urine of persons under stress. Although they acknowledged the fact that ethylvanillin is used as a flavoring agent and gives rise to ethylvanillic acid, they held open the possibility of an endogenous origin of the ethylvanillic acid they detected, based upon the fact that subjects undergoing driving-stress experiments and eating "virtually identical" meals excreted different total amounts and proportions of vanillic and ethylvanillic acids. Our experience on the origin of the 3-hydroxyphenyl acids in urine and a possibility of a relation between

TABLE II. Metabolism of Ethylvanillic Acid.

	Ethylvanillic acid excreted				
Ethylvanillic acid ingested (mg)	Free (mg)	As glucuronide (mg)	As glycine conjugate (mg)	Total (mg)	Total (% of ingested)
100	13.4	21.	1.5	35.9	35.9
500	129.2	61.	23.8	214.0	42.8

their excretion and mental diseases(6) offer a plausible explanation for the results of Hill, *et al.* Although it was possible to assure reasonably similar intake of meals with patients and control subjects, it was difficult to control intake of beverages and refreshments such as coffee, tea, cola beverages, milk shakes, ice cream, sweets, etc., between meals. These foods, unfortunately, represent a major source of chromogenic phenolic substances in urine. Thus, in the driving-stress experiments, even though consumption of meals was controlled, a variable intake between meals could account for appearance of variable total amounts of vanillic and ethylvanillic acids. The varying proportions might be due to the fact that commercial vanilla preparations contain varying mixtures of vanillin and ethylvanillin, and different materials eaten could have been prepared with different flavoring preparations. In the absence of rigorous experimental proof of a new type of metabolic reaction, biological ethylation, it would seem safer to ascribe the appearance of ethylvanillic acid in urine to intake of ethylvanillin.

Summary. Ethylvanillic acid (3-ethoxy-4-hydroxybenzoic acid) occurs occasionally in human urine. Its presence follows ingestion of ethylvanillin, a component of some artificial vanilla flavorings. After ingestion of ethylvanillin or of ethylvanillic acid, free ethylvanillic acid, a glucuronide conjugate, and a glycine conjugate are excreted.

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Multiplication of Single Mammalian Cells in a Nonbicarbonate Medium. (25647)

RALPH B. L. GWATKIN* AND LOUIS SIMINOVITCH†
(With technical assistance of Edmund Bischoff) (Introduced by R. C. Parker)
Connaught Medical Research Labs., University of Toronto, Toronto, Canada

It is standard practice to use a CO_2-bicarbonate system to buffer mammalian cell cultures, even in single cell plating technics where one would expect the other constituents of the medium such as amino acids and serum protein to exert an adequate buffering action. The result of this practice is that cultures must be incubated in a high pCO_2 to control pH. If bicarbonate could be omitted from the medium, incubation could be in air instead of the usual 5% CO_2, and plating out of single cells and manipulation of such cultures would be considerably simplified. This paper describes attempts to attain this objective. It was found that L cells did not form colonies consistently under these conditions unless a Krebs cycle intermediate, oxalacetic acid (OAA), was added to the medium.

Methods. Stock cultures were grown in defined medium CMRL-1066(1) with 20% dialyzed horse serum for the L strain(2) and 20% whole sheep serum for HeLa(3). Both strains were clonal lines. Primary cultures of trypsinized rhesus monkey kidney were grown in Hanks BSS with 0.5% lactalbumin hydrolysate and 1% bovine serum. For the single cell plating experiments, medium with and without bicarbonate was prepared by first making up sterile 2X CMRL-1066 with the bicarbonate omitted. To half of this medium $NaHCO_3$ solution was added to make a 1X medium containing 2.2 g $NaHCO_3$ per liter. The other half was treated similarly except that an equimolar concentration of NaCl was used to replace the bicarbonate. Both of these solutions were then supplemented with bicarbonate-depleted sera at a concentration of 20%. Whole sera could not be used, since loss of CO_2 caused the pH to rise to about 8. Horse serum used for plating the L strain was depleted of bicarbonate by an aseptic dialysis at 4° against 10 volumes of physiological saline, changed twice daily for 3 days. For HeLa and monkey kidney cells, which did not grow in such exhaustively dialyzed serum, bicarbonate was removed by lowering pH of the serum to 6 with 0.1 N HCl and leaving it overnight in a cotton-stoppered flask to allow the CO_2 so liberated to escape. Before use bicarbonate-depleted sera were checked for pH stability on contact with air. A neutralized 0.25 M solution of oxalacetic acid in bicarbonate-free CMRL-1066 was stored frozen and added directly to the Petri dishes. L and HeLa cells were dispersed with 0.035% trypsin (1:300), while a mixture of 0.25% trypsin and 0.05% Versene was used for primary monkey kidney. Dilutions were made in bicarbonate-free CMRL-1066 and 100 cells were added to each dish of medium. The Petri dishes used were 6 cm in diameter and contained 5 ml medium. Cultures with bicarbonate were incubated at 37 ± 0.5° in a flow of 5% CO_2, while nonbicarbonate cultures were incubated in air. Incubation was continued for 2 weeks. In some experiments, as an index of growth after 6-7 days, the cells in 10 randomly selected colonies were counted and averaged. For colony counts the cultures were stained as described previously(4).

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* Present address: Wistar Inst. of Anat. and Biol., Philadelphia, Pa.

† Present address: Dept. of Med. Biophysics, Univ. of Toronto, and Ontario Cancer Inst., Toronto, Canada.

Results. The proportion of cells that formed colonies (efficiency of plating, EOP) varied from 0 to 61% in the bicarbonate-free system (Table I), but with OAA in the medium EOP was consistently high, variation being only what one would expect when plating from cultures grown on glass(4). In addition OAA increased total number of cells which developed after about 1 week of incubation. Those colonies which did develop in bicarbonate-free medium without OAA exhibited cells which were granular and rounded. This necrotic condition was not apparent when OAA was present (Fig. 1). A concentration of 1 to 2 mM OAA was optimum (Table II). Above 3 mM, probably due to spontaneous decarboxylation of the OAA, an appreciable rise in pH occurred. L cells were isolated twice in succession from bicarbonate-free-OAA medium. On replating in the same medium no decrease in EOP or rate of growth was apparent.

TABLE I. Plating Efficiency of L Cells in Bicarbonate and Nonbicarbonate Media Supplemented with Dialyzed Serum. Effect of adding oxalacetic acid to medium in 4 experiments.

Plating efficiency (%)			
Bicarb.*	Bicarb. + OAA†	Nonbicarb.	Nonbicarb. + OAA
43 ± 5‡	73 ± 7	0	83 ± 4
43 ± 12	73 ± 9	0	94 ± 3
		1 ± 1 (50)§	43 ± 15 (285)
		61 ± 4 (40)	125 ± 13 (68)

* Cultures in bicarbonate medium were incubated in 5% CO_2, those in nonbicarbonate medium were incubated in air.

† Oxalacetic acid, 2 mM.

‡ Avg plating efficiency for 3 to 5 plates ± S.E.

§ Figures in parentheses are avg No. of cells/colony after 6 to 7 days incubation.

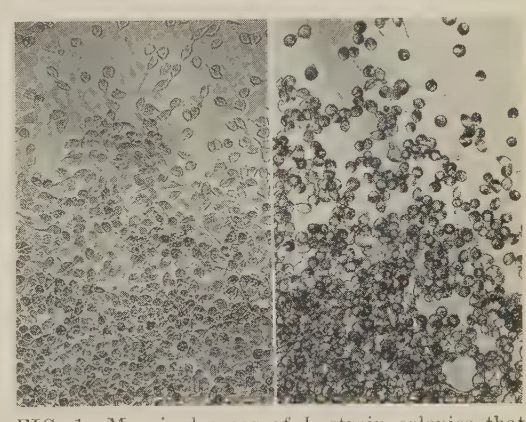

FIG. 1. Marginal areas of L strain colonies that developed in nonbicarbonate medium supplemented with dialyzed serum and equilibrated with air (left) and, under same conditions with 2.5 mM oxalacetic acid (right). Note that abnormal granular condition did not develop when oxalacetic acid was present. (× 90.)

TABLE II. Effect of Varying Concentration of Oxalacetic Acid on Growth of Single L Cells in Nonbicarbonate Medium Supplemented with Dialyzed Serum and Equilibrated with Air.

Conc. of oxalacetic acid (mM)	Plating efficiency (%)	Avg No. of cells/colony after 7 days
.0	0	
.05	0	
.1	0	
.5	35 ± 7*	59
1	53 ± 2	61
2	50 ± 2	69
3	26 ± 6	47

* See corresponding footnote in Table I.

TABLE III. Plating Efficiency of L, HeLa and Monkey Kidney Cells in Nonbicarbonate Medium Supplemented with Serum Selectively Depleted of Bicarbonate by Acid Treatment. Effect of adding oxalacetic acid to the medium.

		Plating efficiency (%)			
Cells	Exp. No.	Bicarb.*	Bicarb. + OAA†	Nonbicarb.	Nonbicarb. + OAA
L	1	59 ±11‡ (74)§	21 ±13 (30)	13 ± 2 (21)	92 ± 3 (65)
	2	13 ± 5 (29)		1 ± 1 (17)	66 ± 4 (50)
HeLa	1	82 ± 3 (26)		52 ± 2 (32)	109 ± 8 (38)
	2	17 ±15 (24)	72 ± 4 (39)	0	14 ± 2 (21)
Primary monkey kidney	1	18 ± 2 (4 mm)‖	35 ± 4 (4 mm)	2 ± 1 (.5 mm)	6 ± 1 (1 mm)
	2	8 ± 2 (3 ”)	18 ± 2 (”)	0	6 ± 0 (”)

*†‡§ See corresponding footnotes in Table I. ‖ Avg diameter of colonies to nearest mm.

The usefulness of OAA-medium for plating other types of mammalian cells was studied using HeLa and monkey kidney. Since these cells failed to grow in exhaustively dialyzed serum, bicarbonate was removed from the serum by acidifying as described under 'Methods'. Sheep serum was employed for HeLa and horse serum for monkey kidney. The results (Table III) suggest that HeLa as well as L cells can be plated out satisfactorily without addition of bicarbonate to the medium provided OAA is present. In contrast monkey kidney cells, which failed to multiply in the bicarbonate-free system, responded only slightly to OAA.

Discussion. It is clear from these experiments that even in a medium containing serum as well as a large number of amino acids and other growth factors a lack of bicarbonate and/or CO_2 interferes with cell multiplication. This agrees with the results others have obtained with bulk cultures of several types of cells(5,6,7). The fact that OAA replaced the CO_2-bicarbonate system for L and HeLa but not for monkey kidney, suggests that CO_2 may be involved in synthesis of different metabolites in these 2 groups of cells. OAA was tested because it substitutes, at least partially, for CO_2 in bacteria, where CO_2 appears to be needed to provide the requisite amount of OAA for the Krebs cycle(8). However, it is well known that CO_2 participates in other areas of metabolism, *e.g.*, in RNA synthesis (9). It would be of interest to study the effect of RNA derivatives, which are not present in CMRL-1066, on ability of monkey kidney cells to multiply in nonbicarbonate media.

In all 3 types of cells, CO_2 and OAA sometimes had an additive effect. A possible reason for this may be that dispersing and diluting tend to arrest the Krebs cycle by washing intermediates from the cells. Under these conditions an intermediate such as OAA may be required in addition to CO_2 to get the cycle started. Neumann and McCoy(10) have shown recently that single cells of Walker carcinosarcoma 256 require Krebs cycle intermediates which are not needed by bulk cultures.

Summary. The feasibility was explored of using nonbicarbonate media, equilibrated with air, for plating single mammalian cells. L strain mouse cells, with which most of the experiments were done, multiplied erratically under such conditions and cell morphology was abnormal. Satisfactory plating could be obtained by substituting oxalacetic acid for the CO_2-bicarbonate system.

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Failure of Acute Bilateral Adrenalectomy to Influence Brain Serotonin Levels in the Rat. (25648)

Jack C. Towne and Joseph O. Sherman (Introduced by Howard L. Alt)
Radioisotope Service, Vet. Admin. Research Hospital and Dept. of Biochemistry, Northwestern University Medical School, Chicago, Ill.

Recently, De Maio reported differential increases of serotonin concentration in pooled fractions of the base, hemispheres, and medulla oblongata of the rat brain 24 hours after bilateral adrenalectomy or hypophysectomy (1). His report suggests a possible influence of endocrine function on brain serotonin metabolism. It was considered of interest to repeat the studies with the acute, bilaterally adrenalectomized rat using a chemical, fluorimetric procedure instead of a bioassay method. Since the surgical procedures employed by De Maio are well known to activate the pituitary-adrenal cortex system, it was considered important to include a sham operated group of animals in order to distinguish specific from nonspecific effects of ablative surgery. In addition our estimations have been made on the individually treated animals, and the results subjected to standard statistical analysis.

Methods. Two studies were made on the effect of bilateral adrenalectomy on brain serotonin concentration. In the first experiment 15 albino rats (Sprague-Dawley strain), weighing between 150 and 200 g, were divided into 3 groups: control, sham operated, and bilateral adrenalectomized. Ether was used as anesthesia in all of the operations. The animals were sacrificed by decapitation 24 hours after surgery. The brain was immediately removed, weighed, and homogenized in 3 volumes of 0.1 N HCl. Serotonin was assayed according to the indirect method of Weissbach, Waalkes, and Udenfriend, involving extraction with butanol(2).

Results. The control group exhibited a mean concentration of 0.65 μg of serotonin per gram of fresh brain tissue. Mean values found in the sham operated and adrenalectomized groups were 0.61 and 0.67 μg of serotonin per gram of fresh brain tissue, respectively. Values for groups of animals were not significantly different from each other.

The above experiment was repeated with addition of a fourth group injected with 100 mg of iproniazid/kilo of body weight 18 hours prior to sacrifice. As in the first experiment, there was no significant difference in brain serotonin concentrations of control, sham operated, and bilateral adrenalectomized groups. The iproniazid treated group showed a 165% increase in brain serotonin over that of control group ($P = 0.01$). Table I summarizes means, standard deviations, and ranges of the groups tested. The data were analyzed by use of the Mann Whitney U-test(3). P values in Table I refer to comparisons of experimental groups to control group. We have also demonstrated that reserpine treated animals have the expected depletion of brain serotonin using the fluorimetric method(4).

To make direct comparison of our data with that of De Maio's, 10 rat brains were separated and pooled into the 4 fractions described by De Maio. Mean weights for these frac-

TABLE I. Serotonin Levels, in $\mu g/g$ Fresh Tissue, of Various Groups under Investigation.

	Control	Sham-operated	Bilateral adrenalectomy	Iproniazid
Mean	.65	.61	.67	
Range	.60–.74	.58–.64	.62–.72	
Stand. dev.	±.047	±.024	±.036	
No. of animals used	5	5	5	
P		>.20	>.20	
Mean	.43	.50	.52	1.14
Range	.37–.51	.35–.63	.40 .66	.99 1.38
Stand. dev.	±.057	±.101	±.092	±.129
No. of animals used	5	5	5	5
P		>.20	>.10	.01

tions and their standard deviations were: 0.41 ± 0.09; 0.26 ± 0.04; 0.32 ± 0.02; and 1.08 ± 0.07 g of tissue for the base, medulla oblongata, cerebellum, and hemispheres fractions, respectively. Recalculating De Maio's data using the mean weights for our pooled fractions, we obtained 0.69, 1.43, and 0.87 μg serotonin per gram of tissue for De Maio's control, bilateral adrenalectomized, and hypophysectomized groups, respectively.

Clearly our results are inconsistent with De Maio's findings. Neither the sham operated nor the bilateral adrenalectomized groups showed a significant increase in brain serotonin level over the untreated group. As expected the iproniazid treated group (which was carried as a control for the reliability of the fluorimetric method) gave the expected significant increase in brain serotonin level (5).

In preparation of brain extracts De Maio used 80% acetone twice for 24 hours. According to Amin, *et al.*(6), use of 80% acetone instead of 95% acetone for preparation of brain extracts greatly enhances the extraction of substance P. Qualitatively the estrus rat uterus responds to substance P in a manner similar to serotonin, but these substances may be distinguished by use of serotonin antagonists(6). It is possible that the apparent increases of serotonin observed by De Maio using bioassay methods were due to substantial increases in substance P and not in serotonin. It is unlikely that closely related derivatives of serotonin could account for the differences observed. Bases which contain the 5-hydroxyindole group would not give a clear response in the bioassay(7), but would contribute towards estimation of serotonin by fluorimetric procedures.

Summary. These experiments find no effect on brain serotonin level as a result of acute bilateral adrenalectomy when the chemical fluorimetric procedure is used for estimating serotonin.

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Adaptation of Vaccinia Virus to Earle's L-Cells with Prolonged Noninfectious Period.* (25649)

CHARLES C. RANDALL AND FRED W. RYDEN
Depts. of Microbiology, University of Mississippi School of Medicine, Jackson, and Vanderbilt University School of Medicine, Nashville, Tenn.

It is well known that vaccinia virus may be cultivated in tissue cultures of various types. Differences in abilities of several strains of the virus to proliferate in established cell cultures have been observed(1). L and LLC-M_1 cell cultures inoculated with Rivers and Minnesota vaccinia virus resulted in initial rounding and clumping of cells, but no morphological changes were noted in succeeding passages and tests for infectivity produced negative results. Previous to this study Scherer(2) made the observation that the Minnesota strain caused "agglutination" of L cells without viral multiplication. The phenomena of agglutination of L cells by vaccinia virus has been investigated further by Mayyasi(3). The present report is concerned with adaptation of a strain of vaccinia virus to L cells by a modification of the usual tissue culture method(4). The emergent strain has been designated VM1.

Materials and methods. Virus: The strain of vaccinia used in this study has been documented previously(1). The stock virus was cultivated in the chorioallantoic membrane of 10-11 day old chick embryos and stored at –45°C; 0.1 ml had an intradermal titer for rabbits of 10^{-5}. *Cell strain*: Earle's L cell strain used in this study was obtained from Dr. W. F. Scherer, Univ. of Minn. All cultures, whether normal or inoculated with virus, received the same nutrient fluid consisting of 40% Earle's salt solution, 20% chick embryo extract and 40% horse serum. *Adaptation experiments*: Two methods were attempted, both at 37°C. The first was the so-called conventional method of blind passage. In the primary trial 0.2 ml of stock virus was introduced into 7 day-old cultures. After incubation at 37°C for 3-4 days the cultures were frozen and ground, diluted 1:10 with their own culture fluid and centrifuged lightly to remove gross cellular debris. In this repetitive experiment 0.2 ml of each proceeding culture was inoculated into succeeding fresh cultures. In the 2nd method (modified procedure) cultures of the same age were inoculated as before but blind passage was not resorted to. Instead, the cultures after inoculation were maintained in the same manner as normal uninoculated ones. *Conventional method*: Within 24 hours following primary inoculation, the cultures showed the characteristic clumping into small masses described by Scherer(2). At 72 hours cells were rounded and granular with some sloughing, and each culture was harvested at this time. By the 3rd passage clumping could no longer be noted. Repetitive passage of material obtained from freezing and grinding the cells through succeeding fresh cultures did not result in demonstrable propagation of the virus as attested by 4 separate trials (10 passages each). Late passage(7-10) ground cells stored at –45°C were not infectious for rabbit skin either by intradermal inoculation or scarification, or for HeLa cell cultures. *Modified procedure*: The changes from 24-72 hours were consistent with those noted above. In 98 hours marked sloughing had ensued and upon gentle agitation most of the remaining cells were detached from the glass. The estimated 5% remaining were round, granular and dark appearing. The supernatant containing the cell debris was aspirated and fresh nutrient added to the parent culture. After several days the remaining cells began to show improvement and in 7 days appeared to be relatively normal with clear cytoplasm and numerous cell processes. In 2 weeks the proliferating cells had largely covered the glass surface. Cells were removed by scraping and transferred to a fresh Carrel flask and were subcultured every 7-10 days. The parent culture was scraped every 12 to 14 days, a portion saved and the remaining cells were dis-

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carded. Representative aliquots from the various procedures were stored at –45°C. Under low magnification (32×) the parent culture and the various subcultures arising therefrom did not appear unusual until the 8th week, when a few scattered gross plaques appeared in both the parent culture and the 6th subculture, but not in control cultures, and clumping of cells into granular masses was prominent. Within 48 hours after onset of plaque formation the remaining cells had largely sloughed from the glass. Cells and culture fluid from previous subcultures (except the first and the 5th, which was lost) or from those scraped from the parent vessel were not infectious for HeLa cells or rabbit skin by scarification or the intradermal technic until onset of cytopathogenic effects. Contamination of the cultures with another strain of vaccinia virus was considered, but can be ruled out for the following reasons. The parent cell culture and the subculture lines were tended by different individuals and identical supernatant was added to these lines and to several different sets of control cultures. It is emphasized that no cytopathogenic changes were observed in normal control cells during or following the study. Subsequent events are worthy of note. Fresh nutrient fluid was added to the few cells remaining adherent to the glass. Daily examination revealed a slow increase in cell population eventually covering the glass surface of the vessel in about 2 weeks. Most of the component cells were clear and did not appear unusual, but scattered cells were granular, rounded and relatively dark, and were interpreted as being infected. When cell population had largely covered the available surface widespread sloughing and necrosis occurred, leaving a few adherent cells which did not appear healthy. This cycle of growth and degeneration was repeated 2 additional times and then terminated. Material from this last procedure (VM1 strain) was supplied to Dr. R. F. Parker, Western Reserve Univ. who did not know of the cycle described above and made the entirely independent observation that infection with the U12 strain of tissue culture cells with VM1 and other strains of vaccinia virus leads to recurring "micro-epidemics"(5). The cycle of infected cell growth and degeneration appears to be similar if not identical to that described here and to studies described by Chambers with EEE virus in L cells(6). The agent obtained by the modified procedure now could be cultivated in usual manner, regularly produced intracytoplasmic inclusions in HeLa cells, L cells and rabbit cornea, and was neutralized specifically by immune sera prepared from 2 other strains(1). The adapted virus now readily infected L cultures and other host-cell systems to approximately the same degree as the highly infectious WR strain(1). Material from the 18th serial passage in L cells revealed an infectious titer of 10^{-4} and 10^{-5} per 0.1 ml for rabbit skin and L cultures respectively.

Discussion. It is significant that the variation in tissue culture method described resulted in adaptation of a strain of vaccinia virus. The explanation is not now apparent although it appears logical that allowing an uninterrupted association of cell and virus permits a more favorable environment for adaptation. During the prolonged non-infectious phase the cells were in contact with complete growth medium so that nutritional depletion of cells was not a factor in latent infection as demonstrated by Morgan with psittacosis virus(7). It is questionable that the present work is an example of latency. It is plausible that a few undetected cells were infected, liberating virus and the end result was adaptation, the mechanism of which is poorly understood. Shope(8) has summarized a number of latent viral infections in tissue culture but the virus was present in pathogenic form capable of detection by infectivity tests either in animal or tissue cultures. It was cited further that in certain persisting infections of HeLa cells with poliovirus the virus did not always exhibit the infectious property. Under certain conditions the latently-infected cells were capable of producing fully active virus.

Finally, the cycles of growth and degeneration described here pose a fundamental problem as to whether an apparently infected cell which presumably has elaborated virus is able to divide. The data do not answer this question but it is curious that the few remaining cells prior to initiation of the cycle of regeneration have the cytological appearance of infection.

Summary. It has been shown with a modification of the usual tissue culture technic that the Minnesota strain of vaccinia virus has been adapted to Earle's L strain fibroblasts. Following establishment of the infection, several cycles of cell growth and degeneration occurred. The adapted strain was identified by conventional cytological and serological criteria. The method described might lend itself to adaptation of other viruses.

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Potentiation of the Local Shwartzman Phenomenon by Trypsin.* (25650)

WILLIAM ANTOPOL AND CHRYS CHRYSSANTHOU
Joseph and Helen Yeamans Levy Labs., Beth Israel Hospital, N. Y.

It has been reported that extracts of bacteria, tumors and normal mammalian tissues produced the Shwartzman Phenomenon in rabbits and hemorrhage into sarcoma 180(1, 2,3) and sarcoma 37(4,5) in mice. It was subsequently found that a number of other preparations of animal, plant and bacterial origin could effect many host responses including the Shwartzman Phenomenon and tumor damaging ability(6,7,8). In these preparations trypsin was employed in the extraction process(7), therefore the final products could contain residual trypsin which was not removed by dialysis. On the other hand we were able to obtain potent extracts, without use of trypsin, from pancreas and testes(2), organs which normally contain proteolytic enzymes(9). Accordingly, it did not seem unreasonable to assume that trypsin *per se* might play a role in production of lesions by bacteria or tissue extracts.

Material and methods. Stock Albino female rabbits were maintained on Rockland rabbit ration pellets which did not contain antibiotics. The abdominal skin was shaved with an electric clipper. Various concentrations of *E. coli* endotoxin (Lipopolysaccharide extract prepared by Difco Laboratories) were injected at different sites in the right flank. The same concentrations in combination with 0.070 mg crystalline trypsin (Takamine Laboratories, Clifton, N. J.) were injected into corresponding sites of the left flank. Other rabbits were treated similarly with *P. vulgaris* polysaccharide(1) instead of *E. coli* endotoxin. A provocative intravenous injection of 0.5 mg *E. coli* endotoxin was administered 24 hours later and the skin reaction was read as in the classical Shwartzman Phenomenon. All solutions were made with pyrogen free saline; the final volume injected in each site was 1 cc. The trypsin solutions were cultured and were not used if found to be contaminated by bacteria.

Results. In a characteristic experiment, in 16 out of 20 rabbits, *E. coli* endotoxin or *P. vulgaris* polysaccharide in combination with trypsin evoked a stronger skin reaction than was produced without use of trypsin. In 10 animals at least one concentration of the bacterial extract was positive in combination with trypsin but did not produce any reaction at the site without added trypsin (Fig. 1). Various concentrations of bacterial extracts in the remaining 6 animals produced skin reaction

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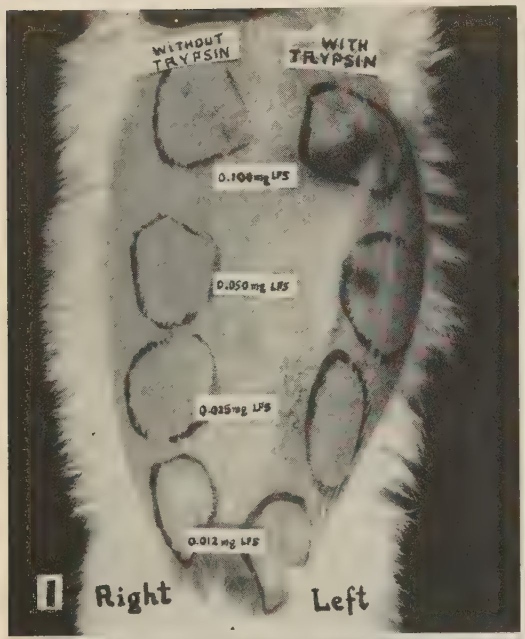
* Aided by grant from U.S.P.H.S.

both with and without trypsin, but in combination with trypsin the reactions were considerably stronger (Fig. 2). In no instance did the site injected with endotoxin alone show a stronger reaction than the corresponding site where endotoxin was administered in combination with trypsin. Various amounts of sterile trypsin up to 1 mg in 1 cc saline solution injected intradermally alone in 15 rabbits did not cause a positive Shwartzman reaction.

Table I indicates that both *E. coli* endotoxin and *P. vulgaris* polysaccharide in concentrations varying from 6 to 25 γ, in combination with trypsin, produced a greater number of positive reactions than the corresponding injection of the endotoxin without added trypsin. From a total of 50 sites injected with endotoxin in combination with trypsin, 36 produced positive reactions and 14 remained negative. From the 50 corresponding sites injected with endotoxin alone, only 24 showed a positive reaction, and 26 remained negative. (A Chi-square test applied to these frequencies reveals that the differences between the compared groups are significant at the 0.02 level of confidence. The same significance is obtained if the percentage of positives is compared — 72% in the group with trypsin *vs.* 48% in the group without trypsin.) In the overall comparative evaluation, *P. vulgaris* extract in combination with trypsin produced positive reactions at 2 concentrations lower than when injected alone; with *E. coli* endotoxin this usually occurred at one concentration lower.

Discussion. It is apparent that trypsin potentiates the effect of skin preparatory substances in elicitation of the local Shwartzman Phenomenon. This indicates that in interpreting the results obtained with trypsin treated substances, it is necessary to equate the effect of the basic substance *per se* with that of the substance in combination with trypsin so that potentiating or activating roles



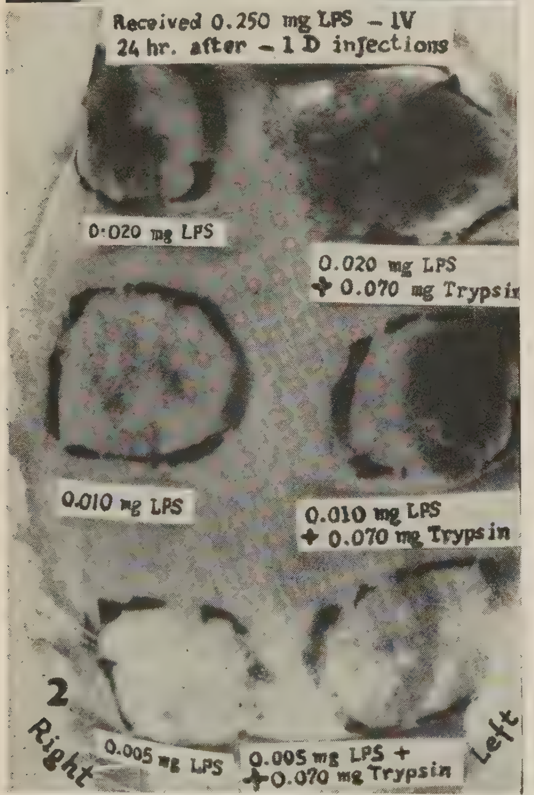


FIG. 1. Left of figure (right flank). Concentrations of *E. coli* endotoxin without trypsin showing negative reaction. Right of figure (left flank). Two corresponding concentrations (.100 mg and .050 mg) in combination with trypsin show positive reaction.

FIG. 2. Left of figure (right flank). Concentrations of *E. coli* endotoxin without trypsin showing weakly positive reaction. Right of figure (left flank). Corresponding concentrations of *E. coli* endotoxin in combination with trypsin showing stronger positive reaction. Potentiation by trypsin is more apparent with .010 mg of endotoxin (center sites).

TABLE I. Potentiation of Local Shwartzman Reaction by Trypsin.

	.025 mg intraderm.			.012 mg intraderm.			.006 mg intraderm.		
		Positives			Positives			Positives	
	No. of cases	Without trypsin	With trypsin	No. of cases	Without trypsin	With trypsin	No. of cases	Without trypsin	With trypsin
E. coli endotoxin	9	5	8	8	3	4	2	0	0
P. vulgaris polysaccharide	10	9	10	11	6	9	10	1	5
Total	19	14	18	19	9	13	12	1	5

might be differentiated and evaluated. The importance of this is exemplified in other reactions which are potentiated or activated by trypsin(10-15).

It is also worthy of note that heparin, which inhibits the Shwartzman Phenomenon(16) also inhibits action of trypsin and serum tryptase(17).

Summary. 1) Trypsin potentiates the effect of skin preparatory substances in elicitation of the Shwartzman Phenomenon. 2) In combination with trypsin, the skin reacts to a greater degree and with lower concentrations of the preparatory factor.

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Tissue Culture Bioautographic System.* (25651)

JOSEPH E. GRADY, WILLIAM L. LUMMIS AND CHARLES G. SMITH
(Introduced by J. S. Evans)
Research Labs., The Upjohn Co., Kalamazoo, Mich.

A bioautographic system under agar has been developed for paper chromatographic analysis of cytotoxic agents *in vitro*. Applications of paper chromatography and bioassay methods in fermentation beer analyses are described.

Materials and methods. Eagle's KB line of human epidermoid carcinoma cells was maintained and harvested as reported previously (1). Cells grown in suspension culture provide a more convenient inoculum than bottle cultures and studies of submerged growth will be reported elsewhere. Cell content of bottle cultures was determined as protein concentration(2); suspension culture populations were determined by hemacytometer count.

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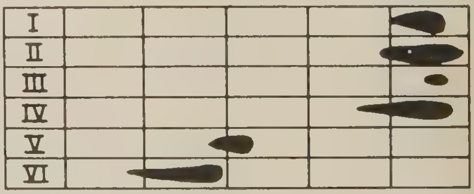
Two milligrams of cell protein (or approximately 4 x 10^6 cells) in 10 ml of Eagle's medium(3) containing 10% calf serum were added to a flat-bottomed Petri dish. Plates were then incubated at 37°C, 80-84% humidity and 8-10% CO_2 in air. Under these conditions, generation time was 18-24 hours. After 18 hours' incubation, supernatant medium was decanted and cell sheet was overlaid with 7 ml of Eagle's medium containing vitamins and amino acids at double strength; adjusted to pH 7 with $NaHCO_3$; 10% calf serum; 0.1% Difco Bacto-Peptone and 0.7% Bacto Noble agar (previously washed exhaustively with distilled water). KB cells under agar had a generation time of approximately 40 hours in above medium. When the agar had solidified, a section of developed papergram was laid on the surface and incubation was continued for 24 hours. When constructing bioautograms it is convenient to divide the chromatogram from origin to solvent front into five equal parts which then can easily fit across Petri dishes. After incubation agar and papergram were floated from the plates in a stream of Earle's salts (minus bicarbonate). Usually, washing the sheet is sufficient to remove cells which have been injured by cytotoxic agents from the chromatogram. When it is desirable to emphasize the zone of inhibition, addition of 10 ml of 0.0125% trypsin in saline and 2-3 minutes' incubation at room temperature is sufficient to cause complete removal of areas of drug-injured cells, since these have a diminished capacity to remain attached to glass and are, therefore, more susceptible to tryptic action than are untreated cells. Experiments with streptovitacin A(4) have shown that the agar technic described above can also be used for disc-plate assay. Aqueous solutions of agent were pipetted onto 13 mm discs which were incubated on the agar surface at 37°C overnight. Agar and discs were removed and the cell sheet was treated as described above before measuring zone size.

Results. Chromatograms of known antitumor agents and fermentation beers were developed in 6 solvent systems listed in Table I. Papergram patterns of actinomycin D with KB cells and *Bacillus subtilis* as test organisms are presented in Figs. 1 and 2. These data show that actinomycin bioactivity is identical when measured in 2 assay systems. Successful papergrams have also been obtained with mitomycin C, streptovitacin family, echinomycin family and fermentation liquors containing unidentified cytotoxic agents. Papergrams of some fermentation products show anti-KB activities while no antibacterial zones can be found. The contrast between "treated" and "untreated" areas must be great to allow quick and definite interpretation of KB bioautographs. This contrast was obtained by the use of a large inoculum as described. If desired, the contrast can be accentuated by fixing the cells with 10% formalin followed by Giemsa stain. Fig. 3 illustrates zones obtained with this procedure.

TABLE I. Solvent Systems Used in Tissue Culture Bioautographic System.

System	Composition
I	n-butanol:water, 84:16 (v/v)
II	n-butanol:water, 84:16 plus 0.25% (w/v) *p*-toluenesulfonic acid
III	n-butanol:acetic acid:water, 2:1:1 (v/v/v)
IV	n-butanol:water, 84:16 (v/v), plus 2 ml piperidine/98 ml butanol:water
V	water-n-butanol 96:4 (v/v)
VI	*Idem* plus 0.25% *p*-toluenesulfonic acid

It should be noted that no inhibition of cells under agar results from application of filter paper *per se*, as evidenced by the confluency of cell sheet on each side of zone of in-



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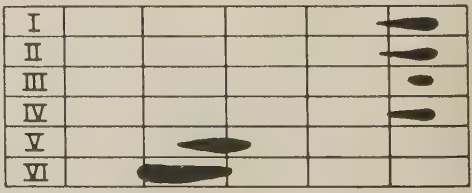

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FIG. 1. Papergram pattern of actinomycin D *vs* KB cells, 1 μg spotted.

FIG. 2. Papergram pattern of actinomycin D *vs* *B. subtilis*, 0.5 μg spotted.

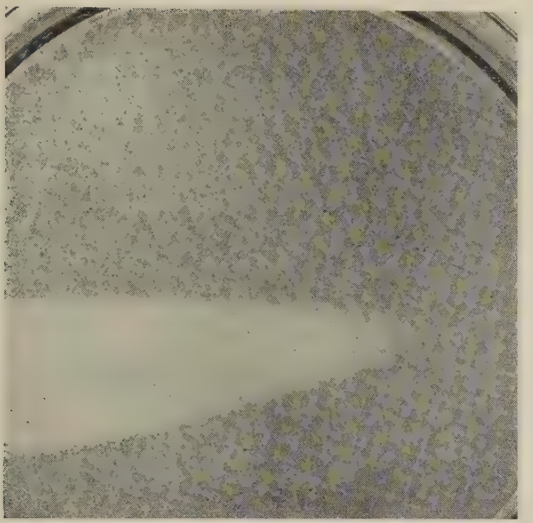

FIG. 3. Photograph of a zone of inhibition obtained by KB bioautograph procedure.

hibition. Thus, the sheet of paper placed on the agar plate shown in Fig. 3 measured approximately 1 x 3 in., whereas the zone of inhibition occurred only where a specific inhibitor was present. Each plate, therefore, serves in part as its own control. The tissue culture bioautogram system provides the only means available to identify unknown cytotoxic agents present in beers and to determine with certainty which activity of a mixture has been removed during fractionation studies.

Disc-plate assay procedure is shown in Fig. 4 with streptovitacin A. As with chromatograms, at least 2 μg of streptovitacin A per disc resulted in complete removal of cells from the surface of the Petri dish giving a zone of at least 18 mm (13 mm disc). Paper discs without agent (controls) did not injure KB cells under agar. If disc-plate assay method can be made as reproducible as tube-assay method(1), it will certainly replace the more laborious procedure (as has occurred with antibacterial and antifungal assays). The technic is most promising and is applicable to determination of chemicals and antibiotics which diffuse in agar. The method is less sensitive than the tube assay however [2 μg per disc can be detected under agar whereas 0.01 μg per ml can be detected in the tube assay(1)].

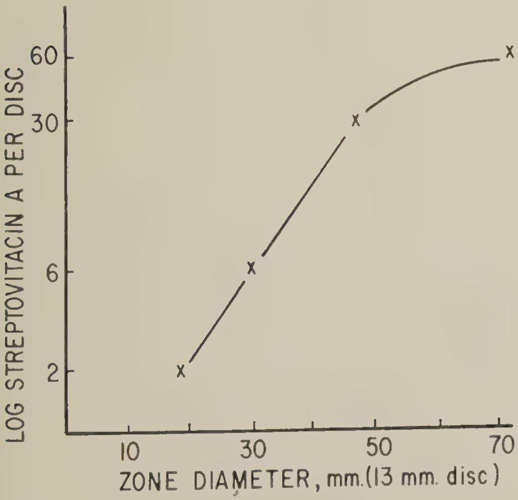

FIG. 4. Effect of streptovitacin A on KB cells in disc-plate assay procedure.

Discussion. The tissue culture bioautograph system has been used in these laboratories to identify cytotoxic activities in fermentation liquors. The technic allows detection of multicomponent cytotoxic activities, identification of components during purification procedures and recognition of identical activities in different beers. An experiment can be completed in 48 hours when the procedure described here is used. If the inoculum is doubled and agar and papergram are added 3-4 hours after inoculation an experiment can be completed in 24 hours. Disc-plate assays and agar papergram systems place screening for potential antitumor agents in the same category as searching for antibacterial or antifungal agents.

Agar-cell systems for assay and chromatography have been described with Ehrlich ascites carcinoma and HeLa cells(5-9) in which dye reduction was used to determine the cytotoxic response. The procedures described here should give a more distinct endpoint determination since dead and injured cells are removed while uninhibited cells remain as a confluent sheet. Cells are growing throughout the period of contact with cytotoxic agents in the KB method, whereas the dye reduction system consists primarily of resting cells. The KB system should be more sensitive with metabolic inhibitors which affect vital processes necessary for growth, division and attachment to glass, since it is entirely feasible that cells in which protein, nucleic acid or energy metabolism is interrupted may be able to reduce a dye but unable to grow. Thus, the KB system is different from those described previously.

Summary. A tissue culture bioautograph system has been described which allows identification of cytotoxic activities on paper chromatograms. Use of this procedure as a disc-plate assay was illustrated and its application in the screening and fractionation of beers with carcinolytic activities *in vitro* was discussed.

The authors acknowledge technical assistance of Lorna Schroeder, Anita Krivis, Wilma Newbre and Marilyn Norman.

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Excretion of α-Ketoglutarate, Urea and Amino Acids by Normal and Muscular Dystrophic Mice.*† (25652)

CHARLES MCGAUGHEY (Introduced by K. Fink)
Vet. Admin. Hospital, Long Beach, Calif.

A preliminary chromatographic survey in this laboratory of urinary constituents of normal and muscular dystrophic strain 129 mice (1)‡ disclosed the presence of a substance which showed a reaction with semicarbazide reagent on chromatograms, and which appeared to be excreted in significantly larger quantities by dystrophic animals than by their littermate controls. The present report concerns identification of the unknown as α-ketoglutarate, further studies of excretion of this keto acid, and a simultaneous chromatographic investigation of amino acid and urea excretion. Amino acid excretion has been found altered or elevated in urines of other animals with various types of muscular dystrophy(2-7).

Methods. Collection and preservation of urine. During urine collection periods, mice were housed in beakers with glass arms fused to the sides at a downward angle to serve as food containers. Access to food (ground Purina Chow) was given by means of holes in the sides of the beakers entering into the side arms. The mice were supported by wide mesh wire screens beneath which were placed at least 3 layers of glass beads pre-coated with thymol by immersion in a thymol-acetone solution followed by draining and drying. This arrangement tended to separate feces and scattered food from urine by allowing the latter to flow to the bottom of the beaker leaving the other materials trapped on top of the beads. Littermate pairs of mice, one dystrophic and one normal, were weighed and placed in the cages in the morning, and after 24 hours feces were removed and urine was collected with several washings. The combined washings were stirred, centrifuged, and supernatants were stored in glass vials at –10°C until analyzed. The urines of 5 pairs of mice were collected over various time intervals and analyzed for α-ketoglutarate, urea and amino

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‡ Obtained from Jackson Memorial Lab., Bar Harbor, Me.

acids as described below. *Identification and analysis of compounds. α-Ketoglutarate.* The chromatographic behavior of the unknown material or its 2,4-dinitrophenylhydrazine (DNPH) derivative (which formed almost immediately at room temperature) was found to be identical with that of synthetic α-ketoglutarate or its DNPH derivative respectively in several different solvent systems(8,9,11). Semicarbazide(9) or DNPH‖ spray reagents were used to detect the free keto acid on chromatograms. Reduction of the DNPH derivative of the unknown by the method of Meister and Abendschein(10) yielded an amino acid, the identity of which was established as glutamic acid by demonstrating that the ninhydrin reacting chromatographic spot given by the unknown amino acid corresponded exactly with a radioautographic spot obtained from glutamic acid-2 C^{14} which had been added to the unknown amino acid as a marker. The amount of radioglutamic acid added was too small to give a detectable ninhydrin spot. Two-dimensional chromatography in 3 pairs of solvent systems and radioautography of chromatograms were carried out as previously described(11). To prepare the DNPH derivative, the reagent was added directly to urine and subsequent extractions of the derivative into ethyl acetate, sodium carbonate and, after acidification of the latter, again into ethyl acetate freed the derivative of contaminating urinary amino acids and excess reagent. Urines were analyzed quantitatively for α-ketoglutarate by a modified method of Bonting and Bonting(12). Reagents were employed at levels 50 times those of the published method(12) and either 0.05 ml or 0.1 ml of collected urine was used. It was shown that certain other unknown materials which appeared on chromatograms after DNPH spray were not visibly extractable from ethyl acetate by sodium carbonate and thus could not have contributed appreciably to the spectrophotometric values obtained for α-ketoglutarate dinitrophenylhydrazone. The absence of detectable levels of α-ketoglutarate in the food and its presence in easily detectable quantities in freshly voided urine were shown chromatographically. *Urea.* This substance was detected on chromatograms run in 80% acetone and sprayed with an acidified alcoholic solution of p-dimethylaminobenzaldehyde (Ehrlich's reagent)(13). If aliquots of the collected urine were diluted 1:10, 5 μl of the resulting solution were usually sufficient to provide a spot of satisfactory intensity for semiquantitative analysis by visual comparison with chromatographed standards. Urea was the only Ehrlich's reacting component detected with this amount of urine. Samples were run in triplicate and standards (1, 2, 3, 5, 7 and 10 μl of 0.01M urea) in duplicate. Each urine spot was evaluated by comparison with each set of standards and 6 resulting figures for each urine sample were averaged. *Amino acids.* These were detected and analyzed by 2 dimensional chromatography and ninhydrin spray as previously described(14). Levels of 100 μl per 8 ml of the collected urine sample were satisfactory for semi-quantitative analysis of the major ninhydrin reacting components. If scattered food was present in a significant amount, corrections were made for the levels of contaminating amino acids contained therein. Although 50-100% errors can sometimes occur for single amino acid measurements obtained by this chromatographic procedure, a large number of samples were run for each mouse, giving a fairly reliable estimate of average excretion over a long period of time. The daily variation in excretion far surpassed the analytical error which was also the case, to a still greater extent, for α-ketoglutarate and urea.

Results. α-Ketoglutarate and urea excretion. Graphs of urinary excretion of these substances per gram body weight together with weights of a representative littermate pair of dystrophic and control mice are shown in Fig. 1. Differences between dystrophics and controls in excretion of α-ketoglutarate and urea became more marked as the disease progressed, and in most cases it was found that even excretion of these compounds *per mouse* was greater by the dystrophic animals during the later stages of the disease.

Data concerning urinary excretion of α-ketoglutarate and urea for all 5 pairs of mice are shown in Table I. Only results obtained between 40 and 105 days were used since, during this interval, there appeared to be no marked changes in excretion as a function of age for any of the mice. A significantly larger amount of both compounds was excreted by dystrophic animals and the differences were often proportionately more marked for α-ketoglutarate than for urea, for which substance differences tended to remain fairly constant. Since a literature search failed to reveal reports of such high levels of α-ketoglutarate in the urines of any animals, urines of 2 other species and 2 other strains of mice were examined for approximate levels of the keto acid. These included Wistar rats, New Zealand white rabbits, CF_1 white mice, and a cross between C3H and strain A mice. Whereas 1-5 μl of undiluted urine from strain 129 mice usually showed a good semicarbazide or DNPH spot in the α-ketoglutarate position on chromatograms, urines from the other animals showed no such spot when chromatographed at levels exceeding those from the strain 129 mice by 4-10 fold. α-Ketoglutarate at high levels was detected chromatographically in the urines of dominant (DyDy) strain 129 mice, though no quantitative studies were done in an attempt to discriminate between this genotype and the heterozygote (Dydy).

‖ 0.05% 2,4-dinitrophenylhydrazine in 2 N HCl.

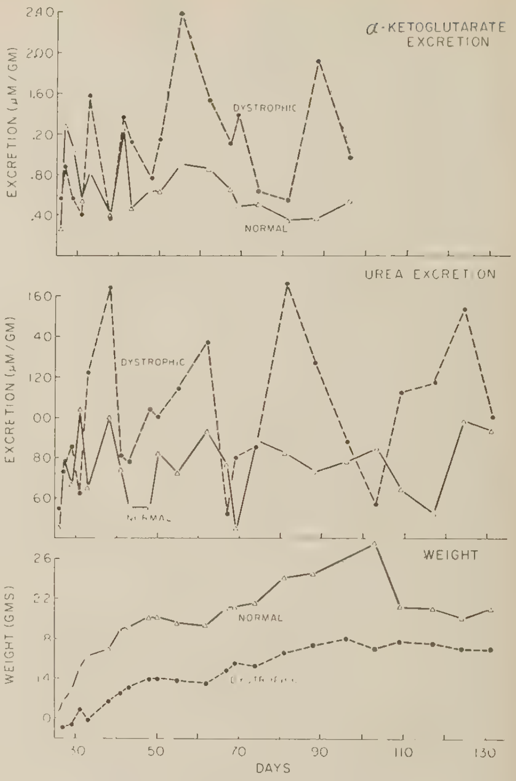

FIG. 1. Urinary excretion of α-ketoglutarate and urea by littermate dystrophic and normal mice.

TABLE I. Urinary Excretion of α-Ketoglutarate and Urea between 40 and 105 Days of Age for Littermate Dystrophic and Normal Mice.

			α-Ketoglutarate Excretion (μM/g/day)			Urea Excretion (μM/g/day)		
Mouse	Sex	Net gain in wt (g)	Mean	Range	No. urines	Mean	Range	No. urines
Dystrophic	♀	3.5	1.23	.38-2.4	11	90	70-170	12
Normal	♀	2.1	.82	.24-2.2	11	60	30-110	12
Dystr	♀	4.8	1.50	.99-2.3	11	120	90-150	12
Normal	♂	10.1	1.00	.49-1.5	11	80	40-120	12
Dystr	♀	2.4	3.00	2.4 -3.8	4	130	70-190	6
Normal	♀	4.0	1.31	.78-2.4	4	100	60-150	6
Dystr	♂	4.5	1.23	.54-2.4	12	100	50-170	13
Normal	♂	8.7	.63	.47-1.3	13	70	50- 90	13
Dystr	♂	10.0	1.40	.26-2.8	20	150	90-210	17
Normal	♂	10.4	.69	.21-2.1	20	110	60-230	17

Cumulative results

	Grand mean	S.D.*	Grand mean	S.D.*
Dystr	1.66	.75	118	24
Normals	.89	.28	86	18
	P† ∼.003		P† ∼.001	

* Stand. dev. † Probability that difference between means is not significant.

Amino acid excretion. The representatives of this class of compounds which were identified by their positions on chromatograms and comprised nearly the total amount of amino acid excretion were: taurine, glutamic acid, glycine, valine, alanine, aspartic acid, methionine sulfoxide, glutamine, phenylalanine, leucine, isoleucine and tyrosine. The listed order is approximately, from greatest to least, that of average daily excretion levels. Taurine was excreted at levels exceeding those of any other amino acid by several fold, the order of magnitude being around 1 μM/g/day. A few other unidentified ninhydrin reacting spots which appeared infrequently were not analyzed. There were no significant differences between dystrophic and normal mice in mean excretion per gram weight of any one or of total amino acids; however, daily excretion of all amino acids except taurine and methionine sulfoxide was consistently and markedly lower (around 50-75%) by 2 of the 5 dystrophic animals than by any of the controls. 1-Methylhistidine was never observed on chromatograms prepared from either normal or dystrophic mouse urines, although this amino acid was the predominant one excreted by rabbits with muscular dystrophy produced by a dietary Vit. E deficiency, whereas their littermate controls rarely excreted detectable levels(7).

Discussion. The consistency of differences in urea excretion between dystrophic and normal littermate mice as opposed to the large variability of differences in weight gain (Table I) tends to suggest that food consumption was fairly random and that variations in the latter probably had a relatively small influence on α-ketoglutarate and urea excretion as compared with the effect of the presence of muscular dystrophy. This is supported by a previous observation(15) that average food intake per unit weight by dystrophic animals was not markedly different from normals.

The increased urinary excretion of α-ketoglutarate by muscular dystrophic as compared with normal mice may have its basis in any of several physiological differences. Among possible reasons already suggested by other work can be included increased transaminase levels in blood and tissues of dystrophic mice (16), increased glutamic dehydrogenase levels in liver and muscle of the pathological animals(17), and increased protein catabolism by dystrophic muscle(18,19) furnishing more glutamate for transamination and deamination. Other metabolic derangements, changed kidney thresholds to α-ketoglutarate, or increased losses of glutamate or α-ketoglutarate from muscle because of increased permeability(20) cannot, however, at this stage be overlooked. The significance of the large excretion of α-ketoglutarate by strain 129 mice as compared with other species of animals and other strains of mice has not yet been assessed.

The elevated excretion of urea by the dystrophic mice could have its origin at least partly in an increased rate of protein catabolism by dystrophic mouse muscle(18,19) as well as an abnormal leakage of nitrogenous constituents from muscle(20) or other tissues. Increased nitrogen excretion has been observed in other forms of muscular dystrophy (21).

The failure in the present studies to observe an altered excretion of amino acids similar to that occurring in other dystrophies(2-7) could have resulted from the large individual variability under the conditions employed. The observation that 2 of the dystrophic animals consistently excreted less of most amino acids than any of the controls suggests an additional possibility that more extensive studies might disclose a deficient amino acid excretion by the pathological animals.

Summary. Unusually large amounts of α-ketoglutarate were excreted in the urines of strain 129 mice as compared with other species of animals and other strains of mice. A group of 5 muscular dystrophic strain 129 mice excreted significantly higher levels of α-ketoglutarate and urea/gram weight than did their 5 littermate controls. These differences became greater as the disease progressed from weaning through about 4 months of age. No significant differences were observed in urinary excretion of amino acids, either with regard to type or amount.

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Toxicity of Sphingosine and Other Long-Chain Amines. (25653)

K. K. Carroll* (Introduced by R. L. Noble)
Collip Medical Research Laboratory, University of Western Ontario, London, Ontario, Canada

Rosenheim and Webster(1) found that feeding cerebrosides promoted conversion of cholesterol to coprosterol in the gastrointestinal tract. They fed various hydrolytic products of cerebrosides and noted that the basic component, sphingosine, could not be tested because the rats refused to eat diets containing either the free base or its sulfate salt. Recently we also observed that basic sphingosine-containing fractions from hydrolyzed cerebrosides were poorly accepted by rats even at very low levels in the diet(2). Further experiments suggested that toxicity was related to presence of free amine groups in these fractions and feeding tests were conducted with a series of simple aliphatic amines. These studies form the basis of the present paper.

Materials and methods. Methods used to prepare psychosine, ceramide, sphingosine and triacetylsphingosine from cerebrosides of beef brain or beef spinal cord have been described (2). Other amines used in present experiments were obtained from Matheson, Coleman and Bell, East Rutherford, N. J. Sulfate salts were prepared by mixing amines with a solution of sulfuric acid in ethanol as in preparation of sphingosine sulfate(3). Volume of ethanol was adjusted according to solubility of individual compounds. Male Sprague-Dawley rats weighing 80-100 g were used for most feeding experiments. Compounds to be tested were added to a synthetic diet consisting of: casein 21, glucose 58, corn oil 10, salt mixture 5 and celluflour 5 parts by weight, with an adequate supplement of water-soluble vitamins but no fat-soluble vitamins. In some experiments corn oil was omitted and glucose increased accordingly. In liquid diets fed by stomach tube, corn oil was retained but casein was replaced by a mixture of egg albumin and lactalbumin (1:3). The dry diet (150 g) was homogenized with water (100 ml) in Waring blendor.

Results. When young male rats were fed a synthetic diet containing 1 or 2% of free aminolipid prepared from beef spinal cord cerebrosides, they lost weight rapidly and

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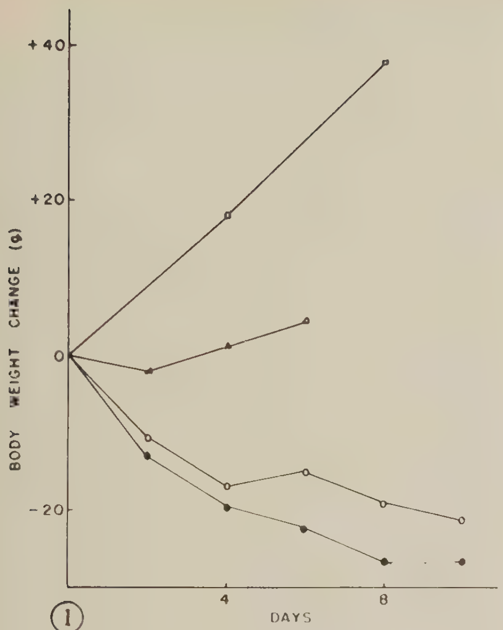

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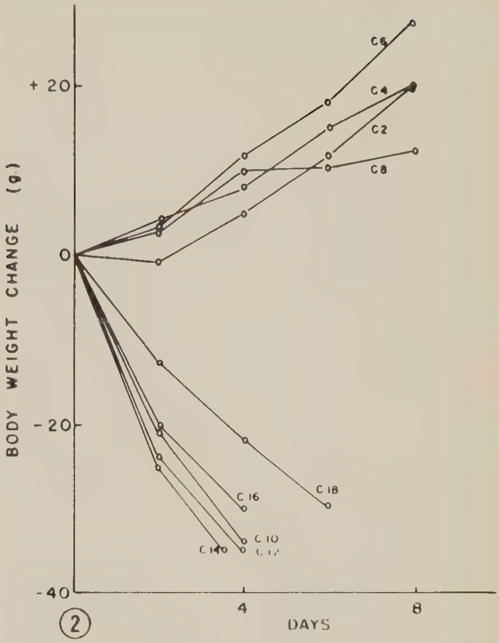

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FIG. 1. Growth curves of rats fed diets containing aminolipid fractions prepared from beef spinal cord cerebrosides. —○— Free base (sphingosine + dihydrosphingosine) as 1% of diet. —●— Free base as 2% of diet. —△— Sulfate salt of base as 1.2% of diet. —□— Triacetyl derivative as 5% of diet. Each curve represents avg for 3 rats.

FIG. 2. Growth curves of rats fed diets containing aliphatic amines as sulfate salts. Amount added in each case was calculated to give a level of 1% of free amine in diet. C 2, ethylamine sulfate, 2.1%; C 4, butylamine sulfate, 1.7%; C 6, hexylamine sulfate, 1.5%; C 8, octylamine sulfate, 1.4%; C 10, decylamine sulfate, 1.3%; C 12, dodecylamine sulfate, 1.25%; C 14, tetradecylamine sulfate, 1.25%; C 16, hexadecylamine sulfate, 1.2%; C 18, octadecylamine sulfate, 1.0%. Each curve represents avg for 2 rats.

usually died after about 2 weeks (Fig. 1). Rats also lost weight rapidly when fed a diet containing 1% of free aminolipid from brain cerebrosides. The latter material is mainly sphingosine whereas the aminolipid from spinal cord cerebrosides is a mixture of sphingosine and dihydrosphingosine(3). The sulfate salt of mixed bases from spinal cord cerebrosides did not depress body growth of rats as much as the free bases and the triacetyl derivative appeared to have no growth-depressing effect at concentrations up to 5% of the diet (Fig. 1).

In these experiments gain or loss in body weight was related to food intake and it seemed that the adverse effects of sphingosine-type bases might be caused by unpleasant taste or odor which made the food unpalatable. To test this, 2 groups of rats were given a diet of fox chow *ad lib.* and in addition animals of one group were given 1 ml/day by stomach tube of a suspension containing 10% by weight of sphingosine[†] in corn oil, while those in the other group were given 1 ml/day of corn oil and served as controls. The group receiving sphingosine ate very little and lost weight while controls showed a continuous weight gain.

In a further experiment sphingosine[†] was incorporated into a liquid diet fed twice daily by stomach tube, thus ensuring that the rats received sufficient food to maintain body weight. Six male rats weighing approximately 130 g were given 5 ml twice daily of the liquid diet without sphingosine and this was increased gradually over 4 days to 10 ml twice daily. After 7 days rats were divided into 2 equal groups and an amount of sphingosine equal to 1% of total solids was added to the diet of one group. Three days later this was increased to 2%. After a further 3 days, animals in the treated group began to appear moribund and it became more difficult to introduce the full 10 ml at each feeding. Autopsy disclosed that the stomachs were very distended with gas and undigested food. The control group continued healthy although did not gain weight rapidly. The results of these experiments suggest that sphingosine exerts a real toxic effect and that the failure of rats to eat diets containing sphingosine is not primarily due to unpleasant taste or odor.

[†] This material was prepared from beef spinal cord cerebrosides and is really a mixture of sphingosine and dihydrosphingosine.

It seemed likely that the amine grouping in the sphingosine molecule was chiefly responsible for the toxic effect. This view was strengthened by feeding experiments with products obtained by partial hydrolysis of cerebrosides. Psychosine, obtained by splitting the amide linkage between fatty acid and sphingosine residues was toxic at the 1% level in the diet while ceramide, obtained by splitting the bond between galactose and sphingosine moieties did not entirely prevent growth when fed at 5% level. It was further found that n-octadecylamine was toxic at the 1% level while n-octadecylalcohol was accepted in the diet at the 5% level. Deichmann *et al.* (4) also describe toxic effects in rats fed diets containing 0.3% of n-octadecylamine.

The degree of toxicity encountered with sphingosine and other long-chain amines was greater than might be expected from comparison with other simple amines. For example, rats gained weight on diets containing as much as 4% of ethanolamine. Because of difference in molecular weights this represents a concentration of amine 20 times greater than 1% of sphingosine. This difference in toxicity between long- and short-chain amines was explored further by feeding a series of simple even-membered amines to rats at the 1% level (Fig. 2). At this level, short-chain amines up to C_8 were accepted and rats grew reasonably well while longer-chain amines from C_{10} to C_{18} were not accepted and rats lost weight rapidly and died. For initial tests the amines were used without purification, but repeat experiments using redistilled octylamine and decylamine gave similar results. However, octylamine appeared to be toxic when fed at the 2% level so the change in toxicity from octylamine to decylamine may not be as abrupt as indicated by Fig. 2.

Discussion. Differences in toxicity between compounds bearing a common functional group may be determined by a number of factors. For example, the compounds may differ in extent to which they interfere with normal metabolic function. They may also be absorbed, transported and excreted in different ways and may be degraded at different rates. Relative toxicities of the degradation products may also play a role.

In aliphatic amines, it is generally assumed that toxicity is due to the amine group and that in animal tissues these compounds are 'detoxified' by oxidative deamination(5). If this generalization holds true, it follows that the substrate specificity of amine oxidases may determine to some extent relative toxicities of different amines. *In vitro* studies have shown that short-chain amines, with the exception of methylamine and possibly ethylamine, are more readily attacked by amine oxidases than are long-chain amines(6,7). However, interpretation of these results is complicated by the low solubility of long-chain amines in aqueous media. Route of absorption of amines may also be important. Long-chain fatty acids are transported from the intestine chiefly *via* the lymphatic system(8) and, if long-chain amines are transported in the same way, they would by-pass the liver, which contains a relatively high concentration of amine oxidase(6,9).

In earlier experiments(2) diets containing 25% or more by weight of cerebrosides supported good growth with no evidence of toxic effects. Since sphingosine and psychosine preparations caused marked growth inhibition at levels of 1% of the diet, it appears that very little free base is liberated during digestion of cerebrosides.

Summary. The free aminolipids obtained by hydrolysis of cerebroside preparations from beef brain or beef spinal cord were poorly accepted by rats at levels as low as 1% of diet. The decreased food consumption and loss of body weight observed when such diets were fed appeared to be related to presence of free amine groups. Long-chain aliphatic amines were more toxic to rats than short-chain amines.

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Some Properties of Rattlesnake Venom Following 26 Years Storage.* (25654)

FINDLAY E. RUSSELL, JERRY A. EMERY AND TRUMAN E. LONG
Lab. of Neurological Research, College of Medical Evangelists, Los Angeles County Hospital

Desiccated extracts of some snake venoms retain their lethality for long periods of time when stored in closed containers in the dark (1,2,3,4). However, certain evidence suggests that some desiccated venoms may deteriorate with time even under optimum conditions of light, vacuum and temperature(3,5). While stability of a venom may be a function somewhat dependent upon the family, genus, or species, lethality and specific modes of action of the venom can be altered by the method of storage.

Studies dealing with toxicity of venoms following periods of long storage are usually confined to determinations of m.l.d. or LD_{50} and give no indication of the changes in zootoxicologic properties. The possibility must be considered that fractions of these complex mixtures may be altered without markedly influencing lethality. The present investigation was initiated to determine the lethal doses of certain rattlesnake venoms stored for 26 to 27 years, and to ascertain the status of several of the known zootoxicologic properties of these venoms(6).

Materials and methods. The venoms were extracted and prepared on the following dates after the methods described by Klauber(6): *Crotalus atrox,* lot 451, July 16, 1932; *C. cerastes,* lot 453, July 16, 1932; *C. mitchelli mitchelli,* lot 510, July 4, 1933; *C. ruber ruber,* lot 496, July 17, 1933; and *C. viridis viridis,* lot 462, Sept. 10, 1932. The intravenous and intraperitoneal m.l.d. and LD_{50} of the dried venoms were determined in 20 g mice as described elsewhere(7). Studies of the effects of the venoms on neuromuscular transmission were made using 15 Bülbring phrenic nerve-diaphragm preparations(8) modified for studies on venoms(9). Studies of cardiovascular, respiratory, and central nervous system effects were made on cats as follows.

In 15 adult cats under pentobarbital hypnosis a common carotid artery and femoral vein were cannulated for measurement of arterial and venous pressures. Bipolar electrodes were placed in the skull over the frontal, parietal, and occipital lobes, bilaterally. Conventional limb, unipolar extremity, and precordial leads were affixed. All electrodes were connected to a Medcraft model D, 8-channel electroencephalograph. Respirations were recorded by means of a cantilever pneumograph(10) fed into the electroencephalograph. In 2 animals intermittent artificial respiration was carried out by positive pressure breathing through a tracheal cannula. Cisternal pressure was measured through a 20-gauge needle fixed in the cisterna cerebellomedullaris, and connected to a modified Brodie tambour in parallel with a water manometer. The tambour activated a wire strain gauge transducer which modulated the ampli-

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* This investigation was supported by grants from Nat. Heart Inst. and Nat. Inst. of Neurol. Dis. and Blindness. The stored venoms were generously supplied by L. M. Klauber. We appreciate comments and criticisms of J. E. Bogen.

TABLE I. Lethality of Crotalus Venoms Following 26 to 27 Years of Storage.

Subspecies	M.L.D. in mg dried venom/20 g mouse Macht	 Githens and Wolff	 Authors	LD$_{50}$ Authors
C. atrox	.12, .30	.12	.20	.22
C. cerastes	.06	.17	.06	.08
C. mitchelli mitchelli	.045	.0035	.005	.008
C. ruber ruber	.11	.22	.12	.14
C. viridis viridis	.045	.025	.040	.045

tude of a 5-cycle carrier feeding the response into the electroencephalograph. A femoral vein was cannulated for subsequent injection of 2.5 to 10.0 mg of one of the venoms dissolved in 2 ml of saline. All animals were heparinized.

Results. Table I shows intraperitoneal m.l.d. in mice of 5 venoms prepared by Klauber in 1932-33, as determined by Macht(11) in 1937, Githens and Wolff in 1939(12), and by us in 1959.

Five mg of *C. mitchelli mitchelli* venom caused complete cessation of the indirectly elicited diaphragmatic contractions in 9 minutes. The blocking capacity in both the stored and fresh toxins decreased in the following order: *C. mitchelli mitchelli, C. cerastes, C. viridis viridis, C. ruber ruber* and *C. atrox,* the last requiring 22 minutes to produce a complete neuromuscular block.

Fig. 1 shows changes produced by the venoms in arterial, venous and cisternal pressures, respiratory rate, and sample electrocardiograms and electroencephalograms. While all venoms produced similar responses, there were individual differences in degree of reaction. These appeared to be dependent upon amount and kind of venom injected. In general, there was a greater variety of responses to these venoms than those freshly prepared and tested in another group of animals(13), and those described by other investigators (14). Following injection of the venom there was an immediate fall in systemic arterial pressure concomitant with an increase in venous and cisternal pressures. Cats receiving artificial respiration displayed similar hypotensive reactions. Apnea of 10 to 45 seconds duration usually occurred after arterial blood pressure had begun to fall. The ensuing respiration was more shallow and rapid, and tended to be abdominal in type. Electrocardiograms showed disturbances in rhythm and ischemic injury patterns. The most common findings were depression of S-T segment, and deviation of T wave from normal. In

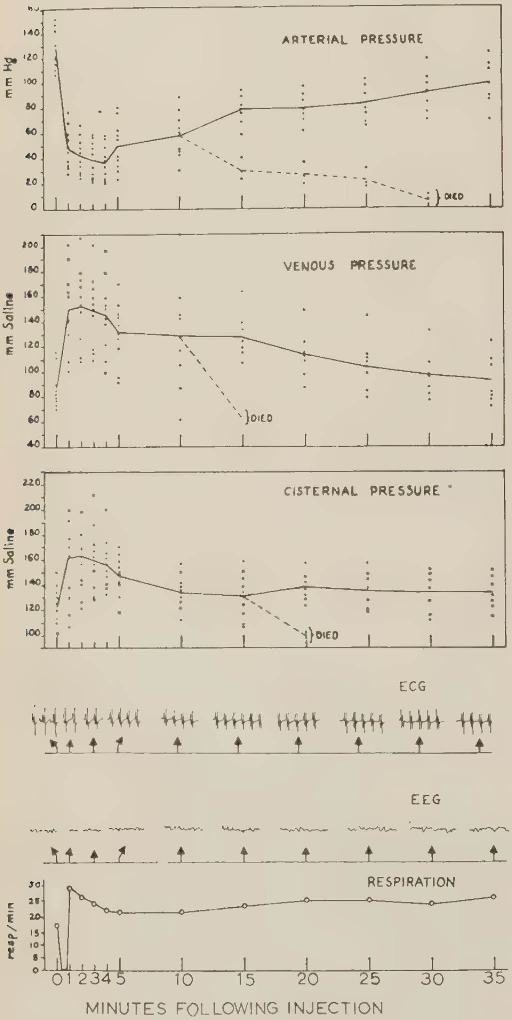

FIG. 1. Effect of stored *Crotalus* venom upon blood and cisternal pressures of 10 cats. A sample ECG (lead II), EEG, and mean respiratory rate for 7 cats are also shown. Inj. at 0 min., 2.5 mg/kg body wt.

some animals no significant changes in electrocardiograms were observed. In 2 cats an ectopic ventricular rhythm developed within 5 seconds following injection and persisted for 10 to 30 seconds. This was followed by sinus bradycardia with pronounced S-T segment shift, and eventually a complete heart block. The electrocardiographic tracings were subject to considerable variation, and while myocardial ischemia, or injury, in some animals may have been due to direct action of the venom on the heart, in the majority of cats the changes appeared to be secondary to the lowered systemic arterial pressure.

Following a sublethal dose of the venom the electroencephalographic tracings flattened; in an occasional animal, random slow waves of varying potentials appeared. The changes occurred during the period when arterial blood pressure was falling. When the point was reached where arterial pressure was beginning to rise and venous pressure to fall, electrical activity over the frontal, parietal and occipital areas began to reappear. Most of the electrical changes could be attributed to changes in blood pressures.

The results indicate that dried rattlesnake venoms stored in closed containers at 42 to 82°F in the dark for 26 to 27 years retained most, if not all, of their lethality, their ability to depress activity at the neuromuscular junction in the phrenic nerve-diaphragm preparation, and the properties responsible for the deleterious changes in arterial, venous and cisternal pressures, respiration, electrocardiogram, and electroencephalogram.

Summary. In mice and cats, dried *Crotalus* venoms stored in closed containers at 42°F to 82°F in the dark for 26 to 27 years retained most of their lethality, their ability to depress neuromuscular junction, and the properties responsible for the deleterious changes in the arterial, venous and cisternal pressures, respiration, electrocardiogram, and electroencephalogram.

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Ability of Some Plasma Protein Fractions to Convert Venous Blood into Hemostatic Blood.*† (25655)

W. O. Cruz, J. R. Magalhaes and Mecia M. Oliveira
Laboratory of Hematology, Instituto Oswaldo Cruz, Rio de Janeiro, Brasil

We have used inability of venous blood to hemostatize the dog hind leg preparation(1) to show the hemostatic activity of many substances, since addition of such substances to venous blood has converted it into hemostatic blood. To investigate which factors in arterial blood were active in hemostasis, since under natural conditions only arterial blood can stop skin bleeding in the hind limb preparation (1), arterial plasma fractionation was performed and protein fractions added to venous blood to detect which of them were active in hemostasis. In this paper, besides the active arterial plasma fractions, other active and inactive fractions obtained from venous plasma and from lymph have been studied.

Methods. All blood samples employed for plasma fractionation or as nutrient for the hind leg preparation were taken from normal mongrel dogs obtained from the animal city pound. Hemostatic capacity of blood samples was tested in the dog hind leg preparation adapted for hemostasis observations(2). In each determination normal arterial blood was used before the sample being studied, to test the preparation for normal bleeding control, and afterwards to confirm the result found in the sample, since it was shown(2) that irrigation of the leg with a non-hemostatic blood sample deprived the preparation of its power to hemostatize, even if normal arterial blood was used afterwards.

Method 10 of Cohn *et al.*(3), *i.e.*, precipitation of protein by buffered alcoholic mixtures of known ionic strength at –5°C, was used, the precipitate spinned down in an adapted refrigerated centrifuge; all reagents were made according to Lever *et al.*(4). The resuspended protein precipitate was tested while as fresh as possible, although fractions could be kept active in the refrigerator for more than a week. Mixing of the fraction with venous blood, kept under petroleum jelly, was carried out just before the test to avoid possible deterioration with time.

Results. Table I shows a marked difference between proteins (from all sources: arterial, venous and lymph) that Cohn labelled I-II-III and the group of proteins called IV-V, as well as a striking difference of behavior of protein fractions from different sources, especially from arterial plasma, citrated lymph, and venous plasma. Except for conflicting results in Fraction IV-V of arterial plasma in which appropriate concentration in 10 determinations corrected the hemostasis of venous blood, and in 6 determinations failed to do so, all other fractions under study showed clear-cut results.

As active fractions were limited to the I-II-III group a further fractionation of this group into I-III and II was performed. The results are presented in Table II. Venous plasma fractions showed no activity in 44 determinations in all 4 groups studied (I-II-III, IV-V, I-III and II) in concentrations varying between 1 to 8 ml in 100 ml venous blood. Separation of active hemostatic proteins by further fractionation of I-II-III has failed, since activity of arterial plasma and citrated lymph fractions was about the same in Fraction I-III and in Fraction II. If we consider the variable results obtained in citrated lymph I-III fraction and the consistent results of Fraction II, it seems that this last fraction is more active, since active concentrations of Fraction II also produced results in agreement with this interpretation.

When an active fraction is added in a much higher concentration than in the range studied (0.25 to 4%) venous blood is no longer converted into hemostatic blood. For instance, Table II shows that with 10% of arterial plasma Fraction II an optimum amount is needed to attain full hemostatic effect.

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TABLE I. Hemostatic Behavior of Fractions I-II-III and IV-V of Lymph, Venous and Arterial Plasmas (Cohn Method 10), when Mixed with Venous Blood.

Fraction	Body fluid used	Fraction conc. in 100 ml venous blood (ml)	No. of determinations	Bleeding time (min.)		
				Previous normal arterial blood	Venous blood plus fraction	Posterior normal arterial blood
I-II-III	Venous plasma	2	3	7 to 8	+30	+20
	Arterial plasma	2	5	5 to 9	5 to 9	6 to 8
	Citrated lymph	2	1	7	7	8
		1	2	7 to 8	6 to 7	10
IV-V	Venous plasma	4	3	6 to 9	+30	+20
		2	3	6 to 10	”	”
		1	4	6 to 10	+20	”
	Arterial plasma	4	3	7	+30	+20
		2	3	7	8 to 9	9 to 13
			3	7 to 8	+30	+20
		1	7	4 to 8	7 to 12	8 to 14
		.5	2	7 to 8	13 to 18	+20
	Citrated lymph	4	4	6 to 8	+30	+20
		2	3	7 to 8	”	”
					21	+30
		1	3	7 to 8	+30	”
		.5	1	7	+20	+20

Discussion. Interpretation of the results depends on behavior of dog plasma in the Cohn alcoholic fractionation. The only paper dealing with dog plasma fractionation by the Cohn method 10 is that of Russ and Raymunt (5). Detailed electrophoretic observations in the Tiselius apparatus were also carried out in our Institute on protein fractions of dog's plasma(6). It was found that the main constituents of dog plasma fractions were much the same as those described in human plasma, except that the greater part of the lipoprotein of dog plasma migrates with alpha globulins and not with beta globulin as in human plasma. Further qualitative differences were observed, such as an unusual distribution of dog beta lipoproteins and the impossibility with the Cohn method of obtaining from dog plasma a pure gamma globulin in Fraction II (a 50% contamination with beta globulin was consistently found).

As Fraction I-III is devoid of gamma globulin and is as active as Fraction II, the hemostatic substances seem to be connected with the only common factor in both of these sub-fractions, *viz.,* beta globulins and beta lipoproteins.

As beta globulin contaminating Fraction II contains cholesterol and phospholipids (10 to 20% of total lipids(7)), a lipoprotein could be the active protein involved in hemostasis.

The fact that substances able to convert venous into hemostatic blood, found in Fraction I-II-III, originated from fractionation of arterial blood and citrated lymph but never from venous plasma, regardless of concentration used (Tables I and II), shows that conversion of venous blood into hemostatic blood is not connected with an enrichment of substances previously present in small concentration in venous blood, but by addition or formation of substances found only in arterial blood and lymph, but absent in venous plasma. We were not able to separate the active principles by further fractionation of I-II-III fraction into Fraction I-III and Fraction II, since both of these last fractions showed a comparable hemostatic activity.

Involvement of the lipid moiety of lipoprotein in hemostasis agrees with our previous studies, which showed the activity of a glycero-phosphatide (cephalin) (8) and especially of one of the 4 of its components – phosphatidylethanolamine(9). Lipid participation in coagulation is shown by Poole(10) and still other facts pointing out participation of lipids in hemostasis will be published later.

That a relatively small amount of arterial plasma fraction when added to venous blood is able to convert this non-hemostatic blood

TABLE II. Hemostatic Behavior of Fractions I-III and II of Lymph, Venous and Arterial Plasmas (Cohn Method 10), when Mixed with Venous Blood.

Fraction	Body fluid used	Fraction conc. in 100 ml venous blood (ml)	No. of determinations	Bleeding time (min.)		
				Previous normal arterial blood	Venous blood plus fraction	Posterior normal arterial blood
I-III	Venous plasma	8	5	7 to 12	+30	+30
		4	5	6 to 10	”	”
		2	4	7 to 9	”	”
		1	3	7 to 8	”	+20
	Arterial plasma	4	4	6 to 10	6 to 9	7 to 8
		2	4	6 to 7	7 to 12	8
		1	3	7	7 to 9	6
		.5	3	6	8	7
		.25	1		8	
		.10	1		+20	
	Citrated lymph	4	3	7 to 8	7 to 12	7
			5	7 to 8	+30	+30
		2	2	5	7 to 9	7
			6	7 to 8	+30	+30
		1	3	7 to 8	7	7
			1	7	+30	+20
		.5	3	7 to 9	”	”
II	Venous plasma	8	3	6 to 8	+30	+30
		4	4	6 to 7	”	”
		2	5	5 to 8	”	”
		1	2	6	”	”
	Arterial plasma	10	5	7 to 8	+30	+30
		4	12	5 to 10	6 to 9	8 to 10
		2	17	5 to 9	5 to 10	7 to 9
		1	10	7 to 9	6 to 10	7
		.5	2		11	+15
	Citrated lymph	10	2	6 to 7	7 to 11	7 to 8
		4	5	6 to 8	6 to 9	7 to 8
		2	4	6 to 8	6 to 8	8
		1	8	6 to 8	5 to 8	8
		.5	5	7 to 9	8 to 9	6 to 16
		.25	3		7 to 9	7
		.1	3	9	+30	7 to +30

sample into full hemostatic blood, suggests that a small imbalance between plasma proteins could have great significance in hemostasis.

Summary. Only protein fractions of arterial blood and lymph are able to convert venous into hemostatic blood. Protein fractions taken from venous plasma lack this property. Fractions IV-V (Cohn method 10) are inert, the hemostatic substances being distributed in Fraction I-III and in Fraction II. There is some indication that plasma constituent able to convert non-hemostatic venous blood into hemostatic blood is related to arterial plasma or lymph beta-lipoprotein.

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Growth Hormone and Carbohydrate Tolerance in Adrenalectomized Rat.*†
(25656)

NORMAN B. MARSHALL† AND FRANK L. ENGEL

Depts. of Physiology and Medicine, Division of Endocrinology,
Duke University Medical Center, Durham, N. C.

Little information is available on the adrenal requirement for producing growth hormone diabetes in the rat. It was stated earlier that anterior lobe pituitary extract was diabetogenic in the partially pancreatectomized-adrenalectomized rat only when adrenal cortical extract was administered simultaneously with the diabetogenic one(1). However, the significance of this finding is clouded by the fact that the amounts of adrenal cortical extract used in these experiments themselves induced glycosuria. The paucity of information as to diabetogenic effect of purified growth hormone in the adrenalectomized rat prompted the present investigation of adrenal requirement for production of hyperglycemia and glycosuria in the rat. Using a recently published procedure(2), which obviated use of partially pancreatectomized animals, the hyperglycemic influence of purified growth hormone was investigated in the tube-fed adrenalectomized rat maintained with various forms of adrenal cortical supportive therapy.

Materials and methods. Male rats of the Vanderbilt strain, weighing approximately 250 g at start of experiment, were housed in individual metabolism cages in a temperature controlled room maintained between 78-80°F. They were adapted to tube-feeding of a high carbohydrate diet(3) during an initial 5-day period. Thereafter, they received a total of 28.0 ml of diet in 2 equal feedings per day (full tube-feeding) at 8:00 a.m. and 4:00 p.m. On the 8th day of full tube-feeding, bilateral adrenalectomy was performed and the animals were placed into 6 groups which received the following types of supportive therapy: (1) 1% saline *ad lib.*, (2) 0.5 mg DOCA/day, (3) 0.1 mg DOCA/day, (4) 0.25 mg cortisol/day, (5) 0.50 mg cortisol/day, (6) 1.0 mg cortisol/day. All injections were by the subcutaneous route and were continued throughout experiment. On 8th and 9th days following adrenalectomy, baseline values for blood glucose and urinary sugar were obtained. From the 10th through the 13th days following adrenalectomy, each animal received either 2.0 mg of growth hormone/100 g of body weight or a control injection of bovine serum albumin at time of morning feeding. On the 12th and 13th days, blood glucose and urinary sugar values were obtained. This dose of growth hormone regularly induces hyperglycemia and glycosuria in intact tube-fed rats(4). Blood glucose was determined by the glucose oxidase method(5) on tail vein blood obtained 3 hours following injection of growth hormone or serum albumin and tube feeding. Urinary glucose was determined on 24-hour collections by the method of Benedict (6). Completeness of adrenalectomy was determined at end of the experiment by ability of the rats to survive a potassium load and by direct observation at autopsy.

Results. The influence of growth hormone administration on glycemia and glycosuria of adrenalectomized rats receiving various amounts of cortisol supportive therapy is illustrated in Fig. 1. There was no significant difference between baseline values and those obtained following administration of bovine serum albumin in all 3 groups. Growth hormone administration to the animals maintained on 0.25 mg of cortisol/day had no effect on blood glucose level and did not result in glycosuria. However, growth hormone administered to rats receiving 0.5 mg cortisol per day resulted in a significantly greater blood glucose level ($p < .01$) than in the injected control rats. Six of the 7 animals in this group developed a mean glycosuria of 1.04 ± 0.22 g/rat/day during the 4-day growth hormone treatment period.

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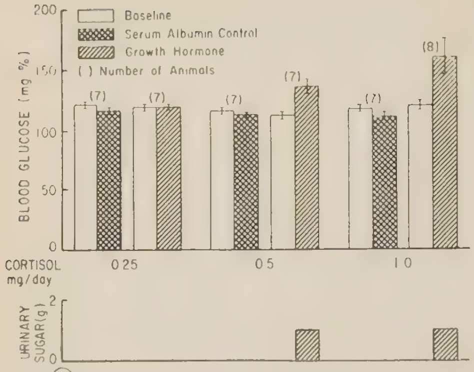
† Present address: Dept. of Nutrition, Upjohn, Kalamazoo, Mich.



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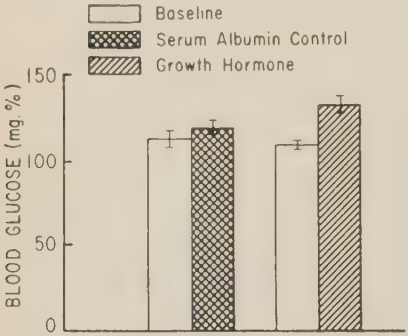

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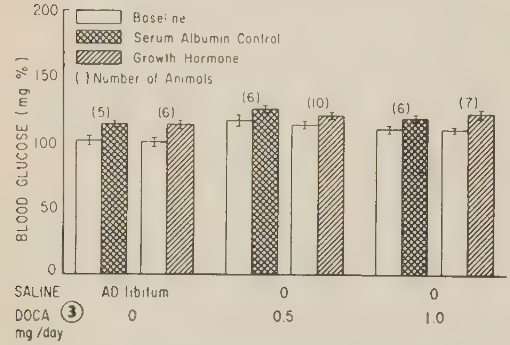

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FIG. 1. Influence of growth hormone on glycemia and glycosuria of adrenalectomized, tube-fed rats maintained on various levels of cortisol.

FIG. 2. Influence of growth hormone on glycemia of adrenalectomized rats after prolonged maintenance on 0.25 mg cortisol/day.

FIG. 3. Influence of growth hormone on glycemia of adrenalectomized, tube-fed rats maintained on saline *ad libitum* or DOCA.

In the group maintained with 1.0 mg cortisol/day and receiving growth hormone, there also was a significant elevation ($p < .01$) of blood glucose over the baseline and the injected control value. Seven of the 8 growth hormone-treated rats in this group exhibited glycosuria during the 4-day treatment period which amounted to 1.0 ± 0.23 g per rat per day.

In both groups with glycosuria, the amount of sugar was slight on the first day of growth hormone administration, reached a maximum on the 2nd and 3rd days and became minimal again on the 4th day.

Administration of growth hormone to the rats receiving 0.25 mg of cortisol was repeated at a time when these animals had received cortisol for a period of 5 weeks (Fig. 2). A small but statistically significant ($p < .01$) elevation of blood glucose concentration was observed, but no glycosuria. This may have been due to the cumulative effect of the microcrystalline suspension of cortisol.

The results of growth hormone administration to adrenalectomized rats receiving either saline or DOCA in doses of 0.5 and 1.0 mg/day as supportive therapy are illustrated in Fig. 3. Growth hormone had no significant influence on blood glucose concentration or urinary glucose excretion under these conditions.

Discussion. The results of this study are consistent with the report that anterior lobe pituitary extract induces glucosuria in pancreatectomized-adrenalectomized rats maintained with adrenal cortical extract, but not saline(1). In the earlier work with anterior pituitary extract the significance of the response was uncertain since the dose of adrenal extract used itself permitted glycosuria. The present study was designed to eliminate this defect by using subdiabetogenic supportive doses of cortisol and, in addition, employed purified growth hormone.

The results obtained support the concept of a species difference regarding the influence of the adrenal cortex and its secretory products in production of growth hormone diabetes. Thus the rat, which requires a glucocorticoid for development of hyperglycemia in response to growth hormone, is unlike the dog, which does not require any adrenal cortical hormonal substitution therapy(7-9), and the cat, which seems to need a functional adrenal gland(10,11). The results with the small dose of cortisol (0.25 mg/day) indicate that magnitude and duration of adrenal substitution therapy are important factors in the rat. Presumably, this may reflect a critical level of adrenal cortical activity which must be attained in the rat to permit the hyperglycemic response to become apparent. In our experiment this may be attributed to an accumulation of depot cortisol over the 5-week period.

The glucocorticoid requirement for growth hormone hyperglycemia in the rat, demonstrated herewith, agrees with data indicating an adrenal steroid requirement for the inhibitory effect of anterior pituitary extract and growth hormone on glucose uptake by the isolated rat diaphragm. Growth hormone injected into adrenalectomized rats has been found incapable of inhibiting glucose uptake by the diaphragm *in vitro* unless the animals were pretreated with cortisone(12-14).

Summary. Growth hormone in a dose which regularly leads to hyperglycemia and glycosuria in normal tube-fed rats has no such effect in saline or DOCA maintained tube-fed adrenalectomized rats. Treatment with subdiabetogenic doses of cortisol restores the hyperglycemic response to growth hormone in adrenalectomized rats. These results are compared to previous studies of growth hormone effects in adrenalectomized cats and dogs.

We are indebted to Upjohn, Kalamazoo, Mich., for hydrocortisone (Cortef) aqueous suspension; to Ciba Pharmaceutical, Summit, N. J., for desoxycorticosterone acetate in sesame oil (Percorten) and to Endocrinology Section, Nat. Inst. Health, for growth hormone.

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Acute Splenic Tumor Produced in Rats by a Mucoprotein Obtained from Rabbit Urine.* (25657)

G. Hodgson,[†] I. Eskuche, S. Fischer and M. Rubio (Introduced by C. C. Congdon)
Inst. de Fisica y Matematicas, Lab. Fisiopatologia, Lab. Parasitologia, Universidad de Chile

When mucoprotein fractions from phenylhydrazinized rabbits' urine were assayed for erythropoietic activity, it was noted that spleens of receptor rats showed marked increase in weight, within 48 hrs. after the first of 2 injections of extracts given one every 24 hours. No correlation was seen between splenic and erythropoietic response, as measured by plasma iron turnover, and uptake of Fe^{59} in erythrocytes. Greatest splenic responses were noted in rats injected with extracts of urine of normal rabbits devoid of erythropoietic activity. Histology of hypertrophic spleens was strikingly similar to that described by Rich(1,2) for acute splenic tumor, and now recognized as a characteristic response of spleen to antigens(3). In view of the magnitude of the response observed in relation to published observations and since it was a response to a soluble antigen, it was decided to study the dose response curve for this extract and its effect on plasma protein synthesis, nucleic acid content and sensitivity to irradiation.

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† Present address: Division of Biol., California Inst. of Technology, Pasadena.

Method. Female grey rats a x c strain (obtained from Inst. de Biologia "Juan Noe" Univ. de Chile) weights ranging from 160-180 g were used. In some experiments fasted rats were used, as this was the condition of animals in assays of erythropoietic activity; in others normal rats were employed. Rats were fasted 3 days and received intraperitoneal injection of the extracts on 2nd and 3rd day of fasting. The rats were then exsanguinated by aortic puncture on the morning of fourth day of fasting; spleens were removed and weighed to the nearest mg; then fixed in Bouin Holland. Histological sections were stained with Hematoxylin Azur II Eosin. Normal receptor rats received either one or 2 intraperitoneal injections of the extracts, one a day and were then sacrificed 48 hours after first injection. Urine was collected overnight from metabolic cages into sterile glass flasks, surrounded by ice-salt sawdust mixture. Urine was centrifuged after collection and then frozen. *Ethanol preparation of extracts.* 1 vol of normal rabbit urine is brought to pH 4.5 with 0.5 M acetic acid, then 4 volumes ethanol are added slowly with constant stirring. The urine is left overnight in the cold; the precipitate is separated by centrifugation, extracted with ether, then dried *in vacuo*. The dry precipitate is extracted with 0.01 M K_2HPO_4, and the material which does not dissolve is separated by centrifuging 20′ at 20,000 g (Lourdes AXJ centrifuge). The supernatant is used in the assays directly or after 24 hours dialysis against 100 vol, 9% NaCl in the cold. Protein content of the supernatant was determined by the Biuret method(4), and the dose injected is given in mg protein. *Amberlite treatment.* In some cases the crude alcohol extract was brought to pH 4.8 by dialysis against 0.01 M acetate buffer. To this solution 20 g Amberlite (IR-45 (OH) Analytical Reagent Fisher Sc. Co.) were added/60 ml extract and the mixture left 48 hr in the cold with constant stirring. The supernatant was then separated and tested for its effect on spleen weight and Fe^{59} distribution (5). *Kaolin adsorption.* 1 vol of urine is brought to pH 4.5 with 0.5 M acetic acid, then 20 g Kaolin (American Standard Acid Washed, Fischer & Co.) is added/liter of urine; the mixture stirred for 1 hr, left at rest for 1 hr, and the precipitate separated by centrifugation. The precipitate is then extracted with 50 ml 1 M NH_4OH/20 g kaolin, the supernatant is separated, brought to pH 4.5 with M acetic acid, then treated with 5 volumes cold acetone ($\sim -4°C$), the precipitate is washed with more acetone, then ether and dried *in vacuo* at 25°C. The dry extract is then treated as in procedure A. *Studies of C^{14} amino acid incorporation into plasma proteins.* 3 μc C^{14} uniformly labelled amino acids (obtained from hydrolysate of chlorella proteins, Radiochemical Centre Amersham, England) was injected intraperitoneally 4 hours before sacrifice of the rats. Blood was obtained by aortic puncture using isotonic citrate-citric buffer at pH 6.4 1 ml/4 ml blood, as anticoagulant. The plasma separated by centrifugation was fractionated with $(NH_4)_2SO_4$ pH 6.4, at different saturations. The fractions collected were those precipitating between 10-30%, 40-50%, and $>$ 50%, that obtained by adding 10% T.C.A. to the supernatant of the 40-50% fraction. The ammonium sulfate precipitates were dissolved in saline, reprecipitated and washed with 10% T.C.A.; all T.C.A. precipitates were extracted with ethanol-ether, then acetone; finally the precipitates were suspended in acetone, filtered on to paper disks, weighed and counted using a thin end-window Geiger counter. A C^{14}-Polyethylene disk obtained from Oak Ridge Nat. Lab., was used as standard for checking the counting system. The results are expressed as C.P.M/mg, corrected for self-absorption using an empirical correction curve. *Irradiation.* Rats were placed in 1 mm wall aluminum, containers 5 x 5 x 15 cm, in a circle at 28.5 cm (mid-line position) from a suspended 130 curie CS^{137}. Ba^{137} γ source (obtained from Oak Ridge Nat. Labs). The exposure dose rate was measured with 100 r thimble ionization chambers, built by Lansdverk Instruments, using a model L 64 roentgen meter. Total dose to which animals were exposed was 550 r, with an exposure of 73.5 minutes. Immediately after irradiation the animals received 6 mg of an ethanol extract of normal rabbit's urine, and 24 hours later another 6 mg. Groups of rats were sacrificed at 2 and 5 days after irradiation. In some irradiated animals, C^{14} AA was injected to study plasma protein radioactivity and others received Fe^{59} for study of plasma iron turnover. The 30-day mortality with 550 r was 3/24 animals in a simultaneously run experiment.

TABLE I. Spleen Weights and Erythropoietic Response of Rats Injected with Extracts of Rabbit Urine and Native Rabbit Plasma. The following abbreviations are used: Et = ethanol; Ex = extract; PH = phenylhydrazine; R = rabbit; N = normal; P = plasma; U = urine. Erythropoietic response was judged by the effects of extracts on Fe^{59} uptake by red cells(5).

Group	No. of rats	Spleen wt (mg)	Erythropoietic response
Control fasted rats	7	290 ± 10	—
Fasted rats + Et Ex PH R U	7	480 ± 20	+
Fasted rats + Amberlite treated Et Ex PH R U	7	262 ± 23.2	+
Fasted rats + Et Ex N R U	14	770 ± 4.1	—
Normal rats	7	310 ± 5.1	—
Idem + Et Ex N R U	7	918 ± 24.5	—
" + N R P	6	301 ± 14.5	—

Results. Table I shows spleen weights in rats receiving 6 mg of an ethanol extract of normal and phenylhydrazinized rabbit urine. The striking increase in spleen weight in animals receiving extracts from normal rabbit urine can clearly be seen. It is interesting to note that injection of 6 ml of normal rabbit plasma in rats produces only very slight changes in spleen weight. Also shown in Table I is the result of amberlite treatment on the splenic effect of ethanol extract of urine of phenylhydrazinized rabbits; the extract maintains its erythropoietic activity, but loses its splenic effect. An example of the histological aspect of the enlarged spleens is given in Fig. 1 which shows marked hyperplasia with abundant large mononuclear cells and frequent mitosis. The D.N.A./g, 146% of controls and R.N.A./g, 160% of controls, content of the enlarged spleens is also strikingly increased, a reflection probably of the increased cellularity. Table II shows a comparison of the effects of different doses of ethanol extracts and the effects of dialyzed ethanol extracts and kaolin extracts of normal rabbit's urine. In experiments summarized here, normal rats were used as receptors and only one intraperitoneal injection of extract was given at 48 hours, after which the animals were sacrificed. It can be seen that the effect on spleen is a function of the dose of material given, that the active factor is non-dialyzable and can be obtained by adsorption on kaolin. Table III summarizes results of experiments in which C^{14} AA uptake in plasma protein fractions was studied. Ethanol extracts of rabbit's urine increase the C^{14} AA uptake in plasma proteins precipitating between 40-50% saturation of ammonium sulfate (α, β globulins). The increase in radioactivity in the 20-33% fraction (γ globulins) is slight, and specific activity of the albumin fraction is similar to that of controls. Table IV summarizes results of radiation experiments. The spleen weight response to ethanol extracts of normal rabbit urine is very markedly depressed after 550 r irradiation. There is no increase in C^{14} AA uptake in plasma protein fractions, and depression of erythropoiesis (Fe^{59} studies) is similar in con-

TABLE II. Dose Response Relation for Splenic Weight Response to Extracts of Normal Rabbit's Urine. D = dialyzed; rest of abbreviations same as in Table I. 6 rats/exp.

Group	Spleen wt
NaCl	320 ± 9.85
1 mg Et Ex N R U	560 ± 5.9
1.5	583 ± 14.6
3	672 ± 18.2
1 D Et Ex N R U	641 ± 11.9
3 kaolin ext. N R U	705 ± 19.2

TABLE III. C^{14} Amino Acid Incorporation into Different Fractions of Plasma Proteins. 6 rats per exp.

Fraction	cpm/mg
20–30% Sat. control	31.5 ± 2.20
" Et Ex N R U	41.5 ± 2.88
40–50% Sat. control	34.9 ± 2.80
" Et Ex N R U	59.2 ± 3.85
>50% Sat. control	33.4 ± 2.90
" Et Ex N R U	35.0 ± 1.12

TABLE IV. Effect of Irradiation on Acute Splenic Response to Extracts of Normal Rabbit's Urine.

			Fe^{59} (3 hr) % dose		C^{14} proteins (cpm/mg)		
		Spleen wt	Plasma	Erythrocytes	20-30	40-50	>50%
Control	2 days post irrad.	184 ± 8.3	52.1	.60	38.85	37	41.2
Et Ex N R U	*idem*	254 ± 9.8	50.9	.60	38.4	35.8	41
Control	5 days post irrad.	192 ± 5.5	43.8	.5	24.9	24.2	23.2
Et Ex N R U	*idem*	243 ± 6.9	32.7	.2	23.9	26.8	23.8
Normal rats		310 ± 5.1	17.4	4.8	31.5	34.9	33.4

trol and treated irradiated animals.

Discussion. The results presented here indicate that a solution containing a few mg of a non-dialyzable, kaolin-adsorbable, ethanol precipitable material probably mucoprotein, obtained from normal rabbit urine, can produce a dramatic increase in rat spleen weight within 48 hours after injection. The active material may be a normal component of urine or a product of bacterial contamination in the bladder; the method used to collect the urine does not allow a distinction to be made between these 2 possibilities. The histologic appearance of the spleen is similar to that described by Rich *et al.*(1,2) as acute splenic tumor, characteristic of the splenic response to foreign protein. Wissler *et al.*(3) have studied rat spleen response to particulate antigens of bacterial origin (Typhoid Friedlander) and sheep red cells. Increases in spleen weight are not as great as those described here, but microscopically similar. The greatest difference is that the injected material in our experiments is soluble, not precipitating at 20,000 g. The fact that rabbit plasma protein, in much larger doses, produces only slight response in rats indicates that the urinary material is much more powerful antigenically than plasma constituents.

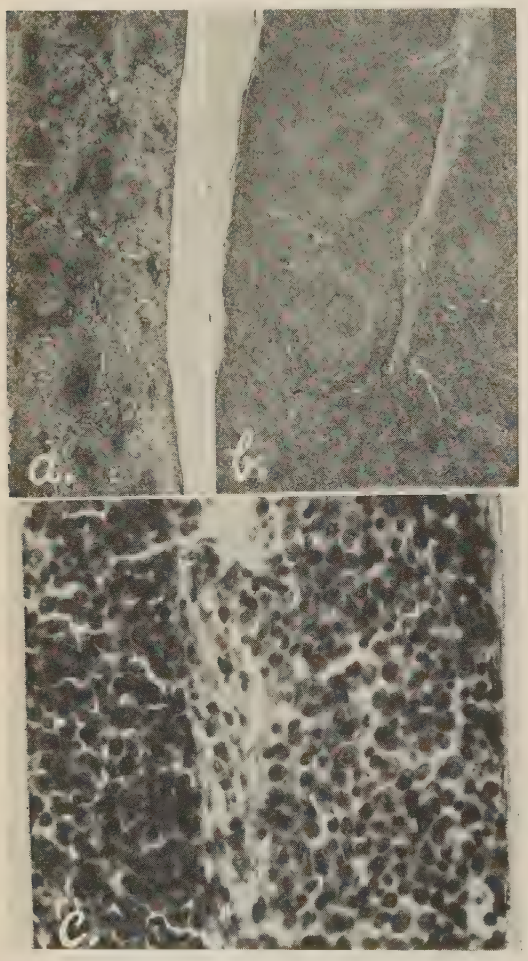

FIG. 1. Histologic aspect of spleen in *(a)* normal rats (× 15), *(b)* spleen of rat inj. with ethanol extract of normal rabbit's urine (× 15) and *(c)* spleen of same rat *(b)* (× 400). Note homogeneous aspect of spleen of treated rats, its great cellularity and presence of many large mononuclear cells.

The finding of an increase in the C^{14} amino acid uptake especially in the plasma proteins precipitating at between 40-50% saturation, (α, β globulin) is surprising in that one would have expected rather to find an increase in γ globulin fraction, which only showed a slight rise. The fact that specific activity of albumin in both control and treated rats was similar, would indicate probably that increased uptake of C^{14} AA in other fractions is a reflection of increased synthesis, rather than of a change in the amino acid pool.

The marked depression of the splenic and disappearance of the plasma protein response caused by 550 r irradiation accords with the finding of others as to the radiosensitivity of the splenic system reacting against antigens (6).

Further purification and chemical characterization of the material responsible for the acute splenic response observed, will be of utility in clearing up the mechanism of the cellular response to purified antigens of one chemical species.

Summary. Extracts of normal rabbit urine obtained by ethanol precipitation or kaolin adsorption produce marked increase in rat spleen weight. Histologic examination of spleens shows marked hyperplasia with abundant mononuclear cells of the type described as acute splenic tumor cells. The splenic response is a function of dose of protein given. Animals treated with these extracts show significant increase in C^{14}-amino acid uptake, specially in the α, β globulin fraction. Preirradiation of the rats (550 r Cs γ) inhibits splenic and plasma protein response to extracts of normal rabbit's urine.

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Effect of BAL (2,3-Dimercaptopropanol) on Hypoglycemic Action of Insulin. (25658)

OSCAR V. DOMINGUEZ* (Introduced by Leo T. Samuels)
Dept. de Farmacologia, E.N.C.B., Inst. Politecnico Nacional, Mexico City, D.F.

Patterson(1) described the diabetogenic action of dehydroascorbic acid (DHAA). Simultaneously, Patterson(2) and Dominguez (3) found that by injecting compounds containing sulfhydryl groups, the animals were protected against the diabetogenic action of DHAA. Intravenous administration of DHAA produces a characteristic response which consists of 2 consecutive periods. The first, a violent convulsive period, starts immediately after intravenous injection of DHAA and is prolonged for no more than 3 minutes. The animals immediately enter the second period, a depressive stage of 5-10 minutes duration. The convulsive period coincides with a deep hypoglycemia. When BAL was injected intramuscularly, the rats were protected against the hypoglycemic response as well as against development of the experimental diabetes induced by dehydroascorbic acid(3). The initial hypoglycemia is probably due to a massive release of insulin from the islets of Langerhans, similar to the release of insulin which has been suggested to follow alloxan injections (4). Since BAL inhibited the initial hypoglycemia produced by DHAA, the effect of BAL on the hypoglycemia produced by exogenous insulin was studied.

Methods. Rabbits of similar weight were divided into 4 groups and fasted for 12 hours before experiment. Blood sugar was determined by Nelson's method(5) and values obtained were considered as normal control levels. Doses of insulin and BAL used in the experiment were 0.7 I.U. and 70 mg/kg of body weight, respectively. The different groups were treated as follows: *Group a* received intravenous injection of insulin. *Group b* received intramuscular injection of BAL. *Group c* were injected intramuscularly with BAL. Thirty minutes later the animals received intravenous injections of insulin. *Group d* received intravenous insulin and intramuscular BAL simultaneously. Blood glucose was

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* Present address: Dept. of Biological Chemistry, University of Utah College of Medicine, Salt Lake City

TABLE I. Effect of BAL (2,3-Dimercaptopropanol) on Insulin Hypoglycemia in Rabbits.

Group		a	b	c	d
No. of rabbits		3	5	6	3
Wt in kg (avg)		2.38	2.52	2.47	2.40
Treatment	Insulin, IU/kg	.7		.7*	.7†
	BAL, mg/kg		70	70 *	70 †
	Time	Blood sugar, mg/100 ml			
	0	112 (104-121)‡	103 (90-115)	102 (75-120)	118 (100-121)
	5 min.				90 (85-107)
	10 "				98 (87-105)
	20 "	98 (85-102)	106 (95-120)	106 (75-129)	65 (58- 70)
	40 "	75 (71- 76)	109 (95-128)	123 (115-140)	85 (70- 95)
	1 hr	63 (60- 67)	108 (90-125)	161 (130-178)	120 (117-135)
	1 ", 20 min.	51 (48- 57)	109 (92-130)	178 (132-225)	173 (165-178)
	2 "	72 (61-101)	115 (95-135)	243 (160-376)	224 (203-249)
	2 ", 20 "	70 (68- 72)	111 (90-135)	255 (185-340)	280 (262-305)
	2 ", 40 "	100 (92-112)	107 (90-128)	265 (192-365)	281 (260-295)
	3 "	109 (103-115)	111 (92-120)	280 (238-360)	291 (265-305)

* BAL inj. (i.m.) 30 min. before insulin. † BAL (i.m.) and insulin (i.v.) simultaneous administration. ‡ Figures in parentheses represent range.

determined every 20 minutes for 3 hours after insulin administration (In Group b, the zero time was the BAL injection).

Results. The results expressed in mg glucose/100 ml of blood are summarized in Table I. These values represent averages observed in the animals studied. The insulin control *Group a* showed the typical fall in blood sugar and recovery to normal values in 3 hours. In *Group b* blood glucose level was practically unaffected by BAL administration. When both BAL and insulin were administered, one would then expect either a depression in blood glucose (if BAL has no effect on insulin hypoglycemia) or continued normal glucose values (if insulin is inhibited by BAL). However, other results were obtained. In *Group c,* not only was the hypoglycemic effect of insulin not observed; a significant increase in blood sugar began 40 minutes after insulin administration (70 minutes after BAL injection), and was still slowly rising at 3 hours.

In *Group d,* the effect of insulin appeared earlier than in the control group but was not so prolonged. A definite hypoglycemia was found at 20 minutes instead of at the usual time, 1 hour to 1 hour, 40 minutes. Within one hour after administration, however, the level had returned to normal and hyperglycemia followed.

Discussion. BAL itself did not significantly affect levels of blood glucose. Perhaps levels of endogenous insulin are so low, that inversion of the response by BAL was not apparent, or perhaps the endogenous insulin and the exogenous crystalline insulin used have different properties.

When both BAL and insulin were injected separately but at the same time (Group d), the hypoglycemic effect of insulin started, but as BAL gradually entered the circulation, inversion of the action of insulin took place. In contrast, when insulin was injected 30 minutes after BAL (Group c), it is probable that some BAL was already circulating at time of insulin injection. This group did not exhibit hypoglycemia and hyperglycemia started sooner than in Group d.

The manner in which BAL produces this effect cannot easily be explained. Huisman (6) found that BAL induced hyperglycemia in rats. Durlacher *et al.*(7), observed that in BAL poisoning, liver glycogen was decreased. Graham(8) found that the hyperglycemic effect induced by adrenaline and noradrenaline was significantly potentiated when BAL, in doses of 25 mg/kg, was previously injected intraperitoneally. The effect of adrenaline was increased 78% and that of noradrenaline 109%. Since insulin releases adrenaline(9), perhaps BAL, when present, inhibits insulin activity on the metabolism of carbohydrates but does not affect adrenaline secretion, so the hyperglycemia occurs. If this is the case, it is necessary to establish that adrenaline is secreted because of the presence of insulin, even when inactivated by BAL.

It is also possible that BAL could act by inhibiting metabolism and inactivation of adrenaline. A further possibility is that BAL increases the release or decreases the metabolism of glucagon. The influence of BAL on the metabolism of glucagon and the catecholamines deserves investigation.

Summary. In rabbits, doses of BAL which had no significant influence on blood glucose levels caused marked hyperglycemia when injected at the same time or 30 minutes before injection of a hypoglycemic dose of insulin. Possible explanations of this effect are considered.

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Interdependence of Hyaluronic Acid and M Protein in Streptococcal Aerosol Infections in Mice.*† (25659)

J. T. CUSTOD, R. I. LYTLE, B. H. JOHNSON AND P. F. FRANK
(Introduced by H. D. Slade)
*Divisions of Biochemistry and Bacteriology, Naval Medical Research Unit No. 4,
U. S. Naval Training Center, Great Lakes, Ill.*

Coburn *et al.*(1) observed that different streptococcal strains administered by aerosol vary in capacity to infect normal mice. Johnson and Furrer(2) confirmed this observation, and also reported that ability of a strain to produce certain levels of hyaluronic acid (HA) appeared related to capacity of the strain to induce aerosol infection. This report stimulated further investigation into the quantitative role of HA production in this phenomenon in presence and absence of M protein, the substance primarily associated with streptococcal virulence. An opportunity to study the latter aspect of the problem was afforded when several strains of group A streptococci were encountered which colonially resembled the aerosol infective organism but which failed to possess M protein as evidenced by serological analysis.

Methods. Streptococcal strains. Twelve typable group A strains exhibiting various kinds of colonial morphology (4 glossy, 2 matt, 5 muco-matt and 1 mucoid) and the 4 non-typable group A strains which colonially resembled the other mucoid strains were all isolated from Navy recruits. *Aerosol technic.* Details regarding construction of the aerosol chamber, culture and preparations of the cell suspensions, spraying technic and reproducibility of the results produced by this technic have been described(2) and are briefly described here. Each streptococcal strain was incubated in Todd-Hewitt broth for 16 hours at 37°C. After centrifugation, the cells were washed 3 times in Gel-Locke solution and final concentration adjusted to a standard optical density value. Each group of 12 Swiss albino mice was sprayed with a streptococcal suspension twice daily for 3 days, then removed from the aerosol chamber, a throat culture taken,

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TABLE I. Effect of Hyaluronic Acid Produced by Group A Streptococcal Strains on Infectivity in Mice by Aerosol.

M type	Colonial morphology	HA production, mg/ml*	% mouse infectivity†
5	Glossy	.0	0 (12)‡
12	”	.0	0 (12)
13	”	.0	0 (12)
1	Matt	.020	41 (12)
3	Muco-matt	.080	75 (24)
3	Matt	.118	67 (12)
6	Glossy	.132	17 (12)
18	Muco-matt	.170	50 (12)
19	”	.188	67 (156)
19	”	.211	” (144)
18	Mucoid	.225	” (12)
1	Muco-matt	.278	100 (12)
NT§	Mucoid	.118	0 (12)
NT	”	.160	0 (12)
NT	”	.165	0 (12)
NT	”	.170	0 (12)

* Avg value of duplicate samples.

† Positive culture from cervical lymph node or lungs of dead and surviving mice.

‡ No. of mice exposed.

§ No reaction with type-specific streptococcal antisera.

and the mice observed for streptococcal death for an additional 10 days. At this time, all survivors were sacrificed and cultured for the strain of beta hemolytic streptococci used in the aerosol. Those mice exhibiting streptococci in cultures of the cervical lymph node were included in calculation of percentage of mice infected found in column 4 of the table. *Hyaluronic acid determination.* To correlate more precisely amount of HA produced and mouse infectivity, the quantitative colorimetric method of HA assay described by Pierce *et al.*(3) was employed in place of the acid-serum method of Seastone used in a previous study(2). Broth culture supernatants of each streptococcal strain grown for 16 hours were obtained by centrifugation at 2500 rpm for 15 minutes. Commercially prepared HA, from umbilical cord,‡ was utilized to prepare a standard curve from which HA values for the broth culture supernatants were determined.

Results. Hyaluronic acid production levels and mouse infectivity data from 16 group A streptococcal strains are shown in the table. Among the 12 typable strains only those producing detectable HA exhibited mouse infectivity. A test of a relationship between the data in columns 3 and 4 of the typable strains only revealed a correlation coefficient of .81 and $P<.01$. Although all 4 non-typable strains produced appreciable amounts of HA, none were capable of infecting mice.

Discussion. Our results confirm the previous findings of Johnson and Furrer(2) that HA production appears to be a required component of group A streptococci for induction of mouse infectivity by the aerosol route. In fact, a significant linear relationship between amount of HA production and infectivity was demonstrated.

Isolation of the 4 group A strains which resembled culturally mouse virulent strains but which differed serologically by failing to demonstrate type-specificity presented an opportunity to obtain information on the role of HA production in infectivity independent of the presence of detectable M protein. Of significant interest was the fact that although all 4 strains produce appreciable amounts of HA, none exhibited mouse infectivity. Of equal importance was the behavior of the 3 typable strains which failed to produce HA and to exhibit aerosol infectivity.

Lack of infectivity in these latter strains could be argued to be due to poor M protein development rather than failure to produce HA since it is conceivable that the quantity of M protein present was sufficient to demonstrate type-specificity but not infectivity. However, it has been reported(2) that several streptococcal strains which type and colonially appear to be rich in M protein, but fail to produce sufficient quantities of HA, exhibited no infectivity when given by aerosol. Therefore, with this evidence in addition to that shown here, it appears that aerosol infectivity of group A streptococci in mice depends on capacity of the strain to produce concurrently HA and M protein.

In view of these results and in contrast to the evidence(4) regarding the minor role of HA in virulence *via* the peritoneal route, there is suggested a different function of HA in establishment of the infectious process by these routes. Just what these differences are cannot be ascertained from the data presented. However, it is postulated that in aerosol infections this muco-polysaccharide material may aid the organism in establishing a foot-hold in the mucosa of the naso-pharynx. It is further reasoned that the M protein is then primarily responsible for the ultimate course of the infection as is the case with streptococcal cells introduced intraperitoneally. Facts tending to support this hypothesis are: a) The dissimilarity of aerosol infectivity and intraperitoneal virulence of a strain as shown by Coburn(5), b) failure of hyaluronidase treatment to appreciably modify virulence of intraperitoneally injected streptococci(4) and c) as shown by this study, the apparent noninfectivity by the aerosol route of streptococci producing hyaluronic acid but not detectable M protein, or of cells producing M protein but not detectable hyaluronic acid.

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‡ Nutritional Biochemicals Corp., Cleveland, O.

Recently Foley *et al.*(6) have reported that virulence of group A streptococci was related to resistance to surface phagocytosis and that this anti-phagocytic capacity was determined by the concomitant presence of large hyaluronic acid capsules and M protein production. Enzymatic destruction of either of these substances resulted in a marked and similar increase in phagocytosis of streptococcal cells by rat leukocytes. It was the opinion of these authors that capsular gel, as well as M protein, plays a significant role in streptococcal pathogenicity. The evidence obtained from our experiment supports their opinion.

Lastly, although there seems to be good evidence that production of HA is essential for aerosol infection, the cause for the high level of HA in broth supernatants of certain highly mouse infective strains has yet to be investigated. Experiments are in progress to determine whether formation of HA is inversely related to hyaluronidase produced.

Summary. Of the 16 group A streptococcal strains tested, only 9 exhibiting type-specificity and production of hyaluronic acid possessed capacity to induce aerosol infections in mice. Conversely, the remaining 7 strains which lacked either one or the other of these attributes failed to demonstrate infectivity by aerosol challenge. A statistically significant relationship between HA production and mouse infectivity was observed.

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Specific Liver and Brain Monoamine Oxidase Inhibition by Alkyl- and Arylalkylhydrazines.* (25660)

A. HORITA AND W. R. MCGRATH (Introduced by T. A. Loomis)
Dept. of Pharmacology, University of Washington School of Medicine, Seattle

Inhibition of the monoamine oxidase (MAO) enzyme of the brain has been demonstrated to result in an increase in brain levels of serotonin (5-hydroxytryptamine) and the catecholamines(1). This increase is believed to be the mechanism by which iproniazid (1 - isonicotinyl - 2 - isopropylhydrazine, Marsilid) exerts its psychic stimulant action. Since these earlier findings with iproniazid other hydrazine derivatives have been investigated for similar anti-MAO activity. Among those tested, the compound β-phenylisopropylhydrazine (PIH)[†] has been found to be an extremely potent inhibitor(2). In addition, Gogerty and Horita(3) demonstrated that PIH administered subcutaneously in graded doses to rats displayed greater brain than liver MAO inhibition, indicating a greater selectivity of the agent for brain tissue. In contrast iproniazid was found to exert an opposite pattern of selectivity; *i.e.*, liver MAO was more effectively blocked, and only by raising the dosage was the brain enzyme affected. These findings were of interest in view of recent reports concerning the possible hepatotoxic effects of prolonged therapy with iproniazid (4,5). The present paper is concerned with investigations to explain the difference between PIH and iproniazid in their relative anti-MAO actions of brain and liver *in vivo*. During these studies a number of related hydrazine derivatives have been employed, and evidence of a relationship between chemical structure and brain MAO inhibition has been found.

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Methods and materials. Male Sprague-Dawley rats weighing 200-300 g were used as the experimental animals. All drugs were injected subcutaneously 2 hours prior to sacrifice of the animals. Livers and brains were rapidly removed, ground with Teflon homogenizers and made up to concentrations of 20% and 33% respectively. All procedures were carried out in an ice bath. One milliliter aliquots of the homogenates were incubated with 4 μmoles of serotonin creatinine sulfate and 0.5 M phosphate buffer at pH 7.0. Total volume of the incubation mixture was 3 ml. All incubations were carried out in a constant temperature shaker at 38°C for 30 minutes (liver) or 60 minutes (brain). Residual serotonin of these mixtures were then extracted and assayed colorimetrically using the nitrosonaphthol method described by Udenfriend *et al.*(6). Degree of MAO inhibition was calculated on ability of the drug to decrease rate of serotonin breakdown under conditions employed. The compounds investigated are shown in Tables I and II. Except for methylhydrazine (Eastman Chemicals) and iproniazid (Hoffmann-LaRoche, Inc.) the hydrazine derivatives were kindly supplied by Dr. J. H. Biel of Lakeside Laboratories, Inc.

Results. In the first series of experiments 4 compounds were studied. Their chemical structures are seen in Table I. In addition to PIH and iproniazid the compounds isopropylhydrazine and l-isonicotinyl-2-phenylisopropylhydrazine (isonicotinyl-PIH) were used. PIH showed marked brain MAO inhibition at a dose of 2.5 x 10^{-6} moles/kg while liver MAO activity was inhibited only to the extent of 25% (Table I). A similar trend was seen at both lower and higher dose levels. In the iproniazid treated rats a greater inhibitory effect of liver enzyme was seen at all dose levels used. Even at doses as high as 10^{-5} moles/kg when liver MAO was inhibited some 75%, brain MAO showed essentially no inhibition. When brains and livers from animals pretreated with isopropylhydrazine and isonicotinyl-PIH were assayed, both agents had produced a greater blockade of liver than of brain MAO. Isopropylhydrazine was found to be a highly potent inhibitor, showing potencies equal to that of PIH; however the pattern of brain and liver inhibition was reversed as compared to PIH, *e.g.*, whereas PIH exerted a 76% brain and 25% liver MAO inhibition at 2.5 x 10^{-6} moles/kg, isopropylhydrazine exerted a 25% brain and a 73% liver MAO inhibition at the same dosage.

These results indicated that the aromatic structure of PIH was important for selectivity for brain MAO *in vivo*. This led to the study of other alkyl and arylalkylhydrazine derivatives, methylhydrazine, benzylhydrazine, ethylhydrazine and β-phenylethylhydrazine. The chemical structures of these compounds are seen in Table II. The purpose of the second series of experiments was to demonstrate the influence of the benzene ring on brain and liver MAO inhibitory effects by the hydrazine compounds. The results are shown in Table II. Both benzyl- and β-phenylethylhydrazine exhibited much greater brain MAO inhibition as compared with the liver enzyme, while their alkyl congeners showed the opposite picture, supporting the results obtained with PIH and isopropylhydrazine. Although the brain-liver

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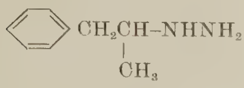
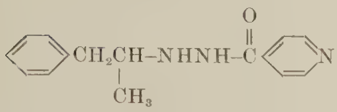
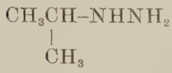
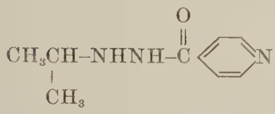
[†] *Beta*-phenylisopropylhydrazine (PIH) is compound JB-516 (Catron), Lakeside Labs.

TABLE I. Liver and Brain Mao Inhibition *In Vivo* by Some Hydrazine Compounds.

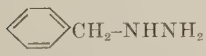
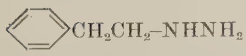
	Dose (moles/kg)	% inhibition Brain	Liver
 PIH	1×10^{-6} (8)	48 ± 9	10 ± 2
	$2.5 \times$ " (6)	76 ± 18	25 ± 6
	$5 \times$ " (4)	98 ± 3	84 ± 3
 Isonicotinyl-PIH	1×10^{-5} (6)	2 ± 3	79 ± 11
	$5 \times$ " (6)	49 ± 4	100 ± 0
 $CH_3\underset{\substack{\vert \\ CH_3}}{C}H{-}NHNH_2$ Isopropylhydrazine	1×10^{-6} (2)	13 (4,22)	43 (29,57)
	$2.5 \times$ " (5)	25 ± 5	73 ± 15
	$5 \times$ " (2)	48 (64,33)	98 (96,100)
 Iproniazid	1×10^{-5} (5)	6 ± 6	74 ± 13
	$5 \times$ " (2)	58 (53,64)	100 (100,100)

Figures in parentheses under dose indicate No. of animals tested at each dose level. % inhibitions are mean values ± their stand. dev. Where n = 1 or 2, actual % inhibitions are placed in parentheses following mean values.

MAO inhibitory pattern of these compounds was related to presence of the benzene ring there was no such relationship in their potencies. Benzylhydrazine was much more effective than its alkyl congener methylhydrazine, while ethylhydrazine showed about a 10-fold greater potency than β-phenyl-ethylhydrazine. In contrast to either of the above pairs both β-phenylisopropylhydrazine (PIH) and isopropylhydrazine exhibited about the same degree of *in vivo* potencies.

Discussion. Gogerty and Horita(3) demonstrated that PIH exerted a more effective inhibition of brain than of liver MAO in the

TABLE II. Liver and Brain Mao Inhibition *In Vivo* by Some Hydrazine Compounds.

	Dose (moles/kg)	% inhibition Brain	Liver
 Benzylhydrazine	2.5×10^{-6} (5)	50 ± 8	21 ± 4
	$5 \times$ " (7)	76 ± 13	34 ± 8
	$7.5 \times$ " (6)	95 ± 7	71 ± 5
$CH_3{-}NHNH_2$ Methylhydrazine	1×10^{-4} (5)	33 ± 5	48 ± 3
	$2.5 \times$ " (2)	63 (66,59)	69 (74,64)
	$5 \times$ " (1)	72	93
 Phenylethylhydrazine	1×10^{-5} (7)	58 ± 16	14 ± 9
	$2.5 \times$ " (4)	96 ± 6	38 ± 9
$CH_3CH_2{-}NHNH_2$ Ethylhydrazine	2.5×10^{-6} (2)	12 (18,6)	25 (25,25)
	$5 \times$ " (5)	35 ± 11	58 ± 7
	$1 \times$ " (5)	64 ± 13	86 ± 3

Figures in parentheses under dose indicate No. of animals tested at each dose level. % inhibitions are mean values ± their stand. dev. Where n = 1 or 2, actual % inhibitions are placed in parentheses following mean values.

intact rat, while iproniazid showed an opposite pattern of inhibition. There are at least 2 possible mechanisms to explain this phenomenon of tissue or organ selectivity by these 2 drugs. The first is that the brain and liver MAO are different enzymes and that they differ in their affinities for drugs. This is, however, unlikely since there is no evidence that brain and liver MAO are different enzymes(7). The fact that in *in vitro* systems both brain and liver MAO are inhibited by iproniazid(8) or PIH(2) suggests that the enzymes are not markedly different. The second possibility is that the chemical structures of the drugs are sufficiently different to permit a different distribution to the various tissues. The brain is especially resistant to passage of certain types of agents because of its well-known but poorly-understood blood-brain barrier. This study supports the view that the chemical structures of the MAO inhibitors are important not only for potency but also for their abilities to exert more or less selective effects on brain MAO.

Upon finding the difference between iproniazid and PIH with respect to brain-liver MAO inhibition *in vivo,* we examined the isonicotinyl derivative of PIH (isonicotinyl-PIH) for its MAO effects. As reported earlier this compound was a much weaker agent than PIH but somewhat more potent than iproniazid(9). In the intact animal it also displayed a greater effect on liver than on brain MAO, thus resembling iproniazid. This finding suggested that the isonicotinyl grouping hindered passage of isonicotinyl-PIH or iproniazid into the brain. When the compound isopropylhydrazine was tested, however, it was found that it also showed a much greater effect on the liver MAO, and brain effects were quite small until much larger doses were used. From the experiments on these 4 compounds, the following has been concluded: (1) iproniazid is composed of 2 structural components which tended to prevent ready access to the brain cells, *i.e.,* the isonicotinyl and the isopropylhydrazine groups; (2) the isonicotinyl group not only hinders brain accessibility but also reduces potency of the compound; (3) the benzene ring of PIH is essential for the drug to exhibit brain selectivity.

The fact that the benzene ring is important for these compounds to exhibit brain selectivity is seen in the experiments with the alkyl and arylalkylhydrazines. In all instances aryl derivatives exerted greater brain than liver MAO inhibition, while alkyl compounds exerted more marked effect on the liver enzyme. It is possible that the aryl compounds, because of the presence of the benzene ring, are more lipoid soluble and therefore pass more readily across the blood-brain barrier. The benzene ring, however, does not alone influence ability of these compounds to exhibit brain selectivity. The presence of certain other groupings may prevent this effect, *e.g.,* with isonicotinyl-PIH, where the isonicotinyl moiety markedly decreased the preferential brain MAO inhibiting property of PIH. This is also true with substitutions of other chemical groupings on the ring, the side chain or the terminal hydrazine group (unpublished results). Of the numerous hydrazine compounds studied thus far it appears that the optimum structure for more selective inhibition of brain MAO *in vivo* consists of an unsubstituted benzene ring, a 2 carbon side chain and an unsubstituted terminal hydrazine group. An alpha methyl group does not affect the brain selectivity, but in fact increases potency of the MAO inhibiting property.

These findings may be of clinical significance since higher brain selectivity of the MAO inhibitors would be desirable for therapy of central nervous system disorders. Compounds such as iproniazid or isopropylhydrazine which act more effectively against liver MAO would thus be expected to exert greater peripheral effects, and for adequate central effects to be produced much higher doses would be required. It is possible that recent reports concerned with hepatotoxic actions of iproniazid during treatment of depression(4,5) may be the result of an excessive effect of this drug on the liver.

Summary. Ability of several hydrazine derivatives selectively to inhibit liver or brain monoamine oxidase (MAO) in the intact rat has been compared. Of those studied, the compounds possessing an unsubstituted arylalkylhydrazine structure, such as benzyl hydrazine, β-phenylethylhydrazine, and β-phenyl-

isopropylhydrazine showed greater brain than liver MAO inhibiting properties. Substitution on terminal hydrazine with an isonicotinyl group reversed the brain-liver MAO inhibiting relationship. Compounds lacking the benzene ring as in methyl-, ethyl-, and isopropylhydrazine also showed greater liver than brain MAO inhibiting effects. It is concluded that the benzene ring of the unsubstituted aryl-alkylhydrazines may aid in their passage into the brain.

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Influence of Local Hypothermia on Absorption from Isolated Intestinal Segments.* (25661)

W. O. Griffen, Jr., A. Castaneda, D. M. Nicoloff, N. H. Stone and O. H. Wangensteen

Dept. of Surgery, University of Minnesota Medical School, Minneapolis

The transport of substances from the lumen of the small bowel to the blood stream under varying environmental conditions has always represented a fascinating physiologic problem. Some effects previously recorded are: interference with vascularity of an isolated loop of bowel slows absorption of strychnine(1); anoxemia increases absorption of water(2); drug-induced decrease of motility of the bowel impedes absorption of radiosodium and heavy water(4), vagotomy which reduces intestinal motility decreases glucose absorption from isolated jejunal loops, while sympathectomy has the opposite effect(5). The reduced intestinal motility(6) attending hypothermia suggested that the effect of hypothermia upon venous return and absorption from isolated canine intestinal loops should be assessed.

Methods and materials. The effect of hypothermia was determined on 1) venous outflow from closed jejunal and ileal loops, 2) strychnine absorption, and 3) removal of radioactive iodine (I^{131}) and radioactive iodinated serum albumin from isolated intestinal loops. Adult mongrel dogs weighing 6-10 kg, anesthetized with intravenous nembutal (30 mg/kg) were used in the study. Through a midline abdominal incision, a loop of ileum or jejunum was delivered through the wound. A 15 to 20 cm segment was isolated between 2 ties of umbilical tape. In the blood flow studies a Y cannula was placed in the mesenteric vein which drained the vascular arcades coming from the obstructed loop. The dogs were given heparin, 1.5 mg/kg. By placing clamps at appropriate sites, the flow could continue into the portal system without obstruction except when interrupted during timed periods (2 minutes) for measuring venous return. In the absorption studies, a polyethylene catheter was placed into the isolated segment through a small enterostomy opening for introduction of materials to be absorbed. When measurement of radioactivity in portal blood was needed, a catheter was threaded through the splenic vein into the portal vein, and 4 cc samples taken at appropriate intervals. In the normothermic (38.5°C) dogs, similar procedures were performed and the segment allowed to remain in

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TABLE I. Small Bowel Blood Flow (ml/Min./kg of Body Wt). 6 dogs.

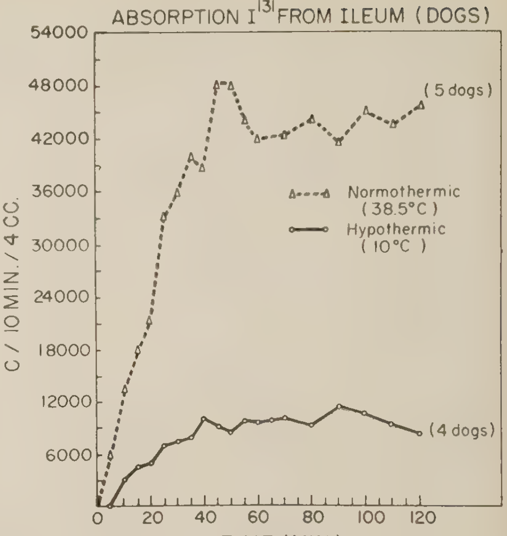
Bowel wall temp., °C	Jejunum	Ileum
38.5	Mean 2.2 Range 1.5–3.2	Mean 1.7 Range 1.2–2.1
14	Mean .72 Range .4–1.4	Mean .47 Range .4– .6

the abdominal cavity. Local hypothermia was accomplished by surrounding the isolated intestinal loop with extremely flaccid balloons through which a coolant solution could be circulated at varying temperatures. A thermistor was placed within the wall of the isolated loop to permit instantaneous recording of its temperature. Body temperature of the animals with local cooling of isolated intestinal segments was maintained at 38.5°C by external heating. In assessing strychnine absorption, 50 or 75 mg of strychnine was placed intraluminally immediately at conclusion of the operation in the normothermic control animals. Strychnine placement in the hypothermic animals was made when temperature of the bowel wall had been reduced to 15°C in some experiments and to 10° in others. In the studies on absorption of the radioactive iodine, 10 μc of RISA or I^{131} were placed intraluminally as in the strychnine experiments. After obtaining a portal blood sample for background counts, collections were begun 5 minutes after the iodine had been placed within the lumen of the bowel and were continued at 5-minute intervals over the first hour, and at 10-minute intervals over the second hour.

TABLE II. Time on Appearance of Convulsions Following Intraluminal Introduction of Strychnine (Jejunum 15-20 cm).

Bowel wall temp., °C	No. dogs	Dose strychnine (mg)	Onset of convulsions (min.)	Animal survival
38.5	4	50	Mean 6 Range 3–8	0
15–20	3	50	Mean 42 Range 35–55	2
10	3	50	No convulsions	
38.5	3	75	Mean 7 Range 3–12	0
15–20	2	75	Mean 38 Range 31–44	0
10	5*	75	Mean 31 Range 18–45	0
25–30†	3	75	Mean 14 Range 5–24	0

* 1 dog did not convulse while the loop was cold.
† Systemic hypothermia.



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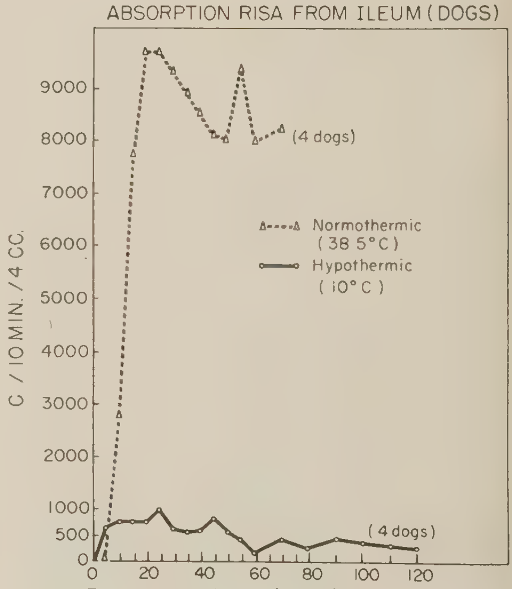

②

FIG. 1 and 2.

Results. Venous outflow: Lowering the temperature of the jejunum from 38.5°C to a level of 14°C (Table I) is followed by a drop in venous outflow in all animals. Mean reduction in similar lengths of jejunum and ileum was 72% and 66% respectively. The difference in mean flow under normothermic conditions may be accounted for by the heavier weight of similar jejunal segments.

Strychnine absorption. Placement of 50 mg of strychnine into a closed jejunal loop at 10°C was not attended by spontaneous convulsions during cooling, but when the normothermic situation was restored, fatal convulsions supervened, (Table II). In contrast, a bowel wall temperature of 15-20°C, while not abolishing convulsions, did permit an occasional survivor. At a dosage of 75 mg, there were no survivors. A jejunal temperature of 10°C prevented spontaneous convulsions in only one dog but quadrupled the time for development of spontaneous convulsions in the others. Systemic hypothermia at the 25-30°C level increases the time period for convulsions to appear, but does not prevent death of the animals.

The 2 factors of decreased venous return and hypomotility during hypothermia probably account for this decreased transport of strychnine. Hypothermic paralysis of cellular enzyme function also may play a part. Experiments using monoiodo-acetamide, a dehydrogenating system poison, in various combinations with strychnine, however, failed to demonstrate any protection from reduced cellular enzyme function.

Iodine and RISA absorption. From Fig. 1 and 2, it is quite evident there is a marked decrease in absorption of I^{131} or radioactive iodinated albumin (RISA) from isolated hypothermic intestinal loops. It is significant that in the normothermic state total absorption of iodine as a free ion is greater than the RISA ($p =< .01$). This indicates some decrease in absorption when the iodine is attached to a semi-permeable molecule, as has been shown to be true for sodium(3). The more effective inhibition of RISA than I^{131} during hypothermia indicates that the absorptive mechanism responsible for the disparity in absorption of free and bound iodine at 38.5°C is markedly affected by cooling.

Conclusions. Our results indicate that hypothermia reduced net transport of substances from gut lumen to blood stream. In strychnine absorption, systemic hypothermia (25-30°C), at clinically feasible levels is not as effective in delaying transport as local hypothermia (10°C). Neither protects the animals from fatal outcome when large doses are used. Hypothermia reduced absorption of RISA approximately ninefold, ($p = .005$) while it reduced absorption of I^{131} only 5-fold ($p = < .01$). Venous return in hypothermic intestinal loops is reduced 72 and 66% in closed jejunal and ileal segments.

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Oxygen Capacity of Stored Frozen Blood.* (25662)

JULIUS SENDROY, JR. AND JOHN D. O'NEAL
Division of Chemistry, Naval Medical Research Inst., Nat. Naval Medical Center, Bethesda, Md.

Principles of rapid freezing and thawing, with especial reference to red blood cells, have been outlined by Luyet(1,2). Following his work, Meryman and Kafig(3,4) prepared, by quick freezing and rapid thawing shortly thereafter, samples of dog and human blood which were, respectively, satisfactory in regard to cell recovery (percentage hemolysis) in the one case, and post-transfusion survival in the other. No storage experiments (with lapse of time between freezing and thawing) were done. The foregoing criteria are ones usually used in evaluating the merits of blood preservation technics. However, the most important function of the red blood cell, namely transport of oxygen to the tissues, is frequently ignored as a measure of the merit of such methods. The following gives results of observations of serial samples of frozen blood stored at low temperatures for periods up to one year. Analyses were done to determine resultant changes, especially in oxygen capacity, which had occurred in the red cell or its hemoglobin (Hb) content.

Materials and methods. The procedure was that described by Meryman and Kafig(4), with recent refinements noted below. Human or ox blood with added ACD solution A (15 ml/100 ml blood) was used. To this mixture (I), was added a 50% *d*-glucose solution (20 ml/100 ml of ACD blood). The final blood mixture (II) was frozen in individual portions of approximately 50 ml delivered into liquid nitrogen from a horizontally mounted syringe, the plunger of which was slowly moved by a screw mechanism. The fluid was forced through vinyl tubing leading to a capillary tube (or 29-gauge hypodermic needle) vertically mounted on a disc rotating rapidly over a liquid nitrogen bath. The frozen particles of the separate portions were stored in individual glass containers (stoppered with gauze plugs) immersed in liquid nitrogen. For reconstitution, liquid nitrogen in the plugged containers was discarded; the frozen particles were thawed by gradual sprinkling from a sieve for 30-45 minutes, into a solution mixture of 12.5 ml 0.9% NaCl and 2.5 ml 50% *d*-glucose, at 40°C. Final volume of the reconstituted blood (mixture III) varied from 52-61 ml. Reconstituted samples were analyzed for O_2 capacity according to Sendroy(5) for both active and total Hb content by the CO capacity method of Van Slyke, *et al.*(6), for cell volume by hematocrit, and for hemolysis by plasma Hb (oxy- and total) content as outlined by Hunter(7). All hemoglobin values were measured as, or converted to equivalent units of, bound O_2 (or CO). Serum potassium was determined by flame photometry (8). Mechanical loss of blood (mixture II) in the freezing and thawing makes quantitatively impossible a controlled, reproducible composition of the final, reconstituted mixture (III). Hence, changes taking place in storage of the samples were evaluated not from absolute values, but from percentage distributions of Hb, as total, carboxy-, and oxy-(6), in "plasma" and cell fractions of each blood mixture (III).† These values formed the basis of comparisons of extent to which physical integrity and Hb content of the erythrocytes, and hence their ability to transport oxygen, had been affected.‡ For comparative

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* The authors are indebted to J. Bronson, G. W. Pearse, and E. Kafig for design and construction of the modified form of apparatus used, and to the latter for aid in preliminary trials of freezing and thawing technic. Determination of hemolysis values by H. A. Collison is gratefully acknowledged. Human and ox blood were obtained through courtesy of the Naval Hospital Blood Bank, Bethesda, and of Dr. A. H. Frank, Agri. Research Service, U. S. Dept. of Agri., Beltsville, Md., respectively.

† "Plasma" refers to non-cell portion of diluted plasma of the final, reconstituted blood (mixture III).

‡ Hemoglobin undergoing denaturation or degradation to a point beyond which it could no longer bind CO upon reduction with $Na_2S_2O_4$(6) would not be measured. However, the results given in the following would seem to make rather small, the likelihood of such an occurrence *i.e.*, any significant absolute reduction in amount of total Hb.

TABLE I. Effect of Rapid Freezing and Storage at –195°C, and Reconstitution of Blood.*

				Blood				Cells		"Plasma"	
Sample mixture	Reconstitution after freezing Days	Hematocrit cell vol %	"Plasma" (Hb)$_{pl}$ Vol %	(Hb)$_{bl}$ Vol %	$\frac{(CO\ cap)_{bl}}{(Hb)_{bl}} \times 100$ %	$\frac{(O_2\ cap)_{bl}}{(Hb)_{bl}} \times 100$ %	Hemolysis $\frac{Hb_{pl}}{Hb_{bl}} \times 100$ %	$\frac{CO\ cap_c}{Hb_{bl}} \times 100$ %	$\frac{O_2\ cap_c}{Hb_{bl}} \times 100$ %	Potassium (K)$_{pl}$ mE/l	$\frac{K_{pl}}{Hb_{bl}} \times 100$
Human II †		39.1	.45	16.86	99.0		1.6	97.4			
III	0	22.9	1.0	10.85	99.0		7.1	92.0			
”	21	26.1	1.4	14.06	98.3		7.4	91.0		28.8	15.1
”	35	29.7	1.6	14.05	98.4		8.0	90.5		28.0	14.0
Ox II †		26.7	.08	13.37	100.0	100.0	.44	99.6	99.6	7.56	4.14
III	14	18.7	.54	9.55	100.0	100.1	4.6	95.4	95.5	4.28	3.58
”	28	20.6	.63	10.06	99.6	99.7	5.0	94.6	94.7	3.96	3.13
”	112	17.9	.55	9.61	100.0	100.0	4.7	95.3	95.3	3.86	3.30
”	175	18.9	.84	10.48	99.0	99.3	6.5	92.6	92.8		
”	224	21.4	.58	11.15	99.4	97.7	4.1	95.3	93.7	4.57	3.22
”	287	17.9	.80	9.57	99.4	97.7	6.9	92.5	91.0		
”	329	18.2	1.00	10.24	99.2	96.8	8.0	91.3	89.1	4.29	3.43
”	364	18.7	1.37	10.61	99.6	97.4	10.5	89.1	87.2	4.25	3.26

* Values in parentheses refer to concentrations in components designated by subscripts; open values denote amounts in component fractions (concentration × component fraction).

† Not frozen.

purposes, loss of potassium from cells to plasma was indicated by the ratio (X 100) of meq of K to total hemoglobin (as ml of O_2 or CO) per unit volume of each reconstituted blood sample.

Results are shown in Table I. The first experiment was done on outdated (22 days old) human bank blood. Hemolysis, or relationship of amount of hemoglobin in "plasma" fraction to total in the whole blood, increased from an initial value of 1.6% (mixture II) to 7% on immediate freezing and reconstitution (mixture III). The value of 7-8 ± 0.4 (analytical probable error) % was found on successive observations of samples removed from storage at intervals of 4-5 days, to a terminal time (in storage) of 35 days. By that time, Hb in the active form (by CO capacity) in the blood had fallen from a level of 99.0 to 98.4 ± 0.7 (analytical P.E.) % of the total. As a result of hemolysis and conversion of active to inactive hemoglobin, the relationship of amount of active Hb in the cell fraction to total Hb in the blood fell from 97.4 to 90.5%.

In the second experiment, changes prior to freezing were avoided by use of freshly drawn defibrinated ox blood. Eight samples containing ACD and glucose (mixture II) were frozen and serially removed from storage for reconstitution and analysis at intervals of lapsed time to one year. Table I indicates increased hemolysis from an initial value of 0.44% before freezing to 4.6% for a sample promptly frozen, stored 2 weeks, then thawed and reconstituted. With longer storage, this value gradually increased to 10.5 ± 0.5 (analytical P.E.)% after 1 year. Hemoglobin in active form in the blood fell from the 100% level to 99.5 or 97.5 ± 0.7 (analytical P.E.)% by CO or O_2 capacity, respectively. Corresponding percentages of active Hb remaining in the cell fraction were 89.1 and 87.2%, respectively. As a result of hemolysis and migration of ions from cells to plasma, ratios of plasma K to total Hb decreased to a small extent from an initial value of 4.14 in the unfrozen mixture II, to values varying between 3.58 and 3.13 ± 0.12 (analytical P.E.) for samples stored from 2 weeks to 1 year.

Although no pH values were obtained, it may safely be assumed that the samples studied were at physiological neutrality. Glass electrode measurements on other samples showed human ACD bank blood (mixture I) 8 weeks old to have a pH of 6.52; however, reconstituted (ox) blood (mixture III) freshly prepared, or after freezing and storage for 4 months, had pH values of 7.35 and 7.41, respectively.

Discussion. Since freezing of blood results in a withdrawal of water from solution, and a concentration of salts, it is, in a sense, a dehydration. From the results of Keilin and Hartree(9), Haurowitz(10), Farr, Hiller and Van Slyke(11), and others, changes in the hemoglobin molecule occur on drying. Thus, when more or less purified preparations of hemoglobin are dried from the frozen or liquid state, with oxygen present, intra- or extra-molecularly, the molecule is rendered vulnerable to oxidation as methemoglobin.

As proposed by Meryman and Kafig(3) blood is quickly oxygenated by air during freezing, is stored and reconstituted, with hemoglobin in the form of HbO_2. Nevertheless, as shown by the excellent agreement of CO capacity with total Hb values for ox blood in Table I, little methemoglobin formation takes place. These results agree with the finding that merely freezing and thawing caused no change in activity of oxyhemoglobin solutions(11). It would seem that freezing, *per se,* is not a dehydration in the sense of a physical withdrawal of water. Furthermore, from the long range point of view, the continued presence of reducing enzyme systems within the cell, instantly available on thawing blood from solid to liquid, would be favorable for preventing oxidative formation of methemoglobin.

On the other hand, the gradually increasing discrepancy between CO and O_2 capacities, possibly indicates formation of a hypothetical heme derivative combining with CO but not with O_2(6). Although the loss of 2.6% (of which 0.5% is apparently in the form of methemoglobin) of O_2 capacity far exceeds the probable error of analysis, it represents a relatively unimportant diminution in functional activity of the reconstituted blood.

These changes in CO and O_2 capacity are reflected in corresponding values for cell fractions, and include the larger loss, by hemolysis, of Hb found in "plasma" to the extent of 10.5% (for ox blood) after 1 year's storage at −195°C.

The results for human blood confirm the occurrence of a small, gradual change in CO capacity (methemoglobin formation) as a result of freezing, storage, and thawing. They also show directly, as do values for ox blood inferentially, that hemolysis takes place immediately upon freezing and thawing. The resultant loss in ox blood, on this account, approximately doubles as storage is extended to one year.§

Potassium content of the "plasma" fraction represents the sum of a moiety escaping through the cell membrane from intact erythrocytes, and another, released from hemolyzed cells, as physical properties of the lipoprotein cell membrane are altered by low temperature and concomitant withdrawal of water as ice. Ox blood ratios of total K in the "plasma" to total Hb in the blood, all 2 or 3 times higher than normal, would be of little importance in transfusion practice. These ratios for human blood, approximately 12 times the values in normal, fresh samples, indicate the usual increase of plasma K for blood stored 21 days in the liquid state. There is no reason to attribute any significant change in plasma K to the effect of storage or ageing at −195°C. Indeed, the results for ox blood indicate, with passage of time in the solid state, as in the liquid, an increasing reversal on addition of glucose to mixture II(12), of the initially rapid migration of potassium from cells to plasma(13), in this case offsetting an increasing amount released from hemolyzed cells.

Summary. 1. Blood diluted to increased volume of approximately 40%, has been rapidly frozen and stored for one year, in liquid nitrogen. 2. Periodic examination of serial samples showed a gradual loss of O_2 capacity of 2.5%, of which 0.5% represented conversion to methemoglobin. 3. As a result of freezing and thawing, an immediate hemolysis of 5-6% took place, which was slowly approximately doubled during the year. 4. Accumulation of potassium in plasma as a result of escape from intact cells and liberation from hemolyzed erythrocytes was apparently arrested or nullified by reversed migration of K ions to the cells.

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§ Obviously, the scatter of values for hemolysis at different points of storage indicates that these results (Table I) aside from relatively small analytical errors indicated, are not wholly attributable to age of sample, but reflect a procedural variability incurred in handling (preparation, freezing, storage, and thawing) of individual samples. However, an underlying general trend of increase in hemolysis with time, is quite clear.

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Stimulatory Effects of Glycine, L-Serine, Folic Acid and Related Compounds on Growth of Cell Cultures.* (25663)

ROBERT E. NEUMAN AND ALFRED A. TYTELL
Research Division, Merck Inst. for Therapeutic Research, West Point, Pa.

Twelve amino acids, L-glutamine, and infrequently L-asparagine are required for growth of cell cultures(1). Additionally, primary rhesus monkey testicular[†] and primary monkey kidney cultures(2) require glycine. Isolated HeLa and KB cells(3) and a strain of rabbit fibroblasts(4) require L-serine. Either glycine or L-serine is essential to the Novikoff hepatoma *in vitro*(5) and these amino acids stimulate growth of Walker carcinosarcoma 256(6) and Jensen sarcoma cultures(7).

In this communication the stimulatory effects of glycine and L-serine for a wide variety of both primary and stable line cultures are described and relationships of this requirement to folic and folinic acid are examined.

Methods and materials. Inocula for primary cultures were prepared by stirring the minced fresh tissue 2-6 hours with 0.2% trypsin (Difco 1:250) in Earle's balanced salt solution at 37°C. The cell suspension was filtered through gauze, collected by centrifugation and resuspended in whole medium. Stable line cells for inocula were prepared by trypsinization of stock cultures except Walker carcinosarcoma 256 cells which were readily shaken loose from the glass after chilling at 5°C. One ml of whole medium containing 100,000 fresh cells or 20,000 stable line cells was pipetted into 15 x 125 mm tubes which were placed in a slanted position in racks. The cultures were established (plated) by incubation for 24 hr at 37°C in an atmosphere of 8% carbon dioxide—92% air. The medium was removed and the tubes were rinsed with Earle's solution. The cells then were overlaid with 1 ml of test medium containing graduated amounts of the test compounds. Incubation was continued for 3-5 days until the cells formed a sheet or attained maximal growth in presence of optimal amounts of the test compounds. The medium was not replenished. Growth was measured by the method of Oyama and Eagle(8). The whole medium in which the cells were established was similar to that previously described(9). It contained L-isomers of essential and nonessential amino acids as well as 0.2 mM pyruvate, 10 μg adenosine, and 5% bovine serum. No p-aminobenzoic acid was included in media used. The test medium differed in that it contained dialyzed serum, 0.2 mM each of pyruvate, alpha-ketoglutarate, and oxalacetate.

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TABLE I. Stimulation of Growth of Primary and Stable Line Cultures by Glycine and L-serine.

	Added to basal medium:		
Cultures	Nothing	Glycine	L-serine
	Relative growth (inoculum = 1.0)		
Stable lines:			
HeLa (human tumor)	8.2	9.1	9.1
KB (” ”)	18.0	22.5	26.0
FL (” amnion)	8.0	8.6	9.4
Kidney (Rhesus monkey)	8.4	10.2	9.2
Heart (cynomologous monkey)	13.3	14.8	14.6
Kidney (rabbit)	10.6	11.9	11.4
Walker carcinosarcoma 256 (rat)	8.7	12.3	14.2
Strain L (mouse)	5.0	5.3	5.0
Sarcoma 180 (mouse)	5.5	5.7	5.9
Heart (chick embryo)	15.4	19.1	18.5
Primary cultures:			
Green monkey kidney	4.3	12.9	10.2
Calf kidney	3.4	4.7	4.4
” testis	5.7	8.1	8.1
Rabbit kidney	6.6	14.6	10.1
” lung	3.6	4.9	5.2
” testis	5.2	8.7	8.4
Guinea pig kidney	4.7	6.6	6.1
” ” lung	5.0	11.0	11.0
” ” testis	6.3	13.5	11.8
Hamster lung	4.2	6.9	6.3
Rat lung	5.9	7.6	7.6
” testis	9.3	11.9	10.8
Chick embryo kidney	2.6	6.1	6.8
” ” lung	5.3	7.2	9.6
Cockerel kidney	5.7	7.5	7.7
” lung	8.8	13.6	13.1
” testis	4.4	5.7	7.8

Conditions of growth are described in text. Basal medium contained no glycine or L-serine. These compounds were added in graduated quantities up to 2 mM levels.

Adenosine and glutathione were omitted. Media for growth of avian cultures also contained 6% dialyzed chick embryo extract prepared from equal volumes of 12 day chick embryo and Earle's solution. Dialysis of serum and embryo extract was conducted as described previously(9). Earle's solution was modified in these studies to contain 3.0 g glucose and 6.46 g sodium chloride per liter.

Results. In Table I are shown stimulatory effects of optimal amounts of glycine (0.1-0.2 mM) and L-serine (0.5-1.0 mM) upon growth of a number of primary and stable line cultures. The results are expressed in terms of maximal growth attained relative to 24 hour plated cells. The majority of cultures responded appreciably to additions of glycine or L-serine to the medium. Effects of either compound were detectable at 0.01 mM concentrations. Growth was often depressed by 0.5-1.0 mM glycine and 1.0-2.0 mM L-serine. Although glycine and L-serine were interchangeable in their role as stimulatory nutrients, the influence of one compound was found to be superior to the other in many instances. These differences possibly express interconversion rates of glycine and L-serine. In general, stable line cells were stimulated 10-50%. Only the mouse strain "L" and sarcoma 180 cells failed to respond significantly. The majority of primary cultures were stimulated 50-200%.

Dimethyl glycine and sarcosine at 0.01-10 mM concentrations substituted for glycine only to a slight extent in growth of Walker carcinosarcoma 256 and primary green monkey kidney. Glyoxylic acid was somewhat more active but again response was much inferior to that with glycine or L-serine.

Eagle reported replacement of the glycine requirement of monkey kidney cultures by folinic acid(1). This prompted examination of the effects of folic and folinic acids on glycine requirement for maximal growth of representative cultures. In Fig. 1 are shown relative effects of folic acid, glycine, and L-serine upon growth response of typical cultures. Stimulation by glycine and L-serine is evident. Increased concentration of folic acid (0.5-1 μg/ml) also resulted in slightly improved growth for the majority of cultures although the test medium routinely contained 0.2 μg folic acid per ml. Maximal effect of folic acid usually was attained at 5-20 μg/ml. Folinic acid‡ in experiments conducted simultaneously stimulated primary green monkey kidney and chick embryo lung to a similar degree at 0.01-0.1 μg/ml. With the remaining cultures, 1-5 μg was necessary to produce effects comparable to those of higher concentrations of folic acid. Nevertheless, neither folic acid nor folinic acid potentiated growth of the majority of cultures equal to that of glycine or L-serine. Walker carcinosarcoma 256 was particularly unresponsive to folic and folinic acid. Primary green monkey kidney cells

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‡ Leucovorin, formyl tetrahydropteroylglutamic acid. Lederle Labs, Am. Cyanamid Co., N. Y.

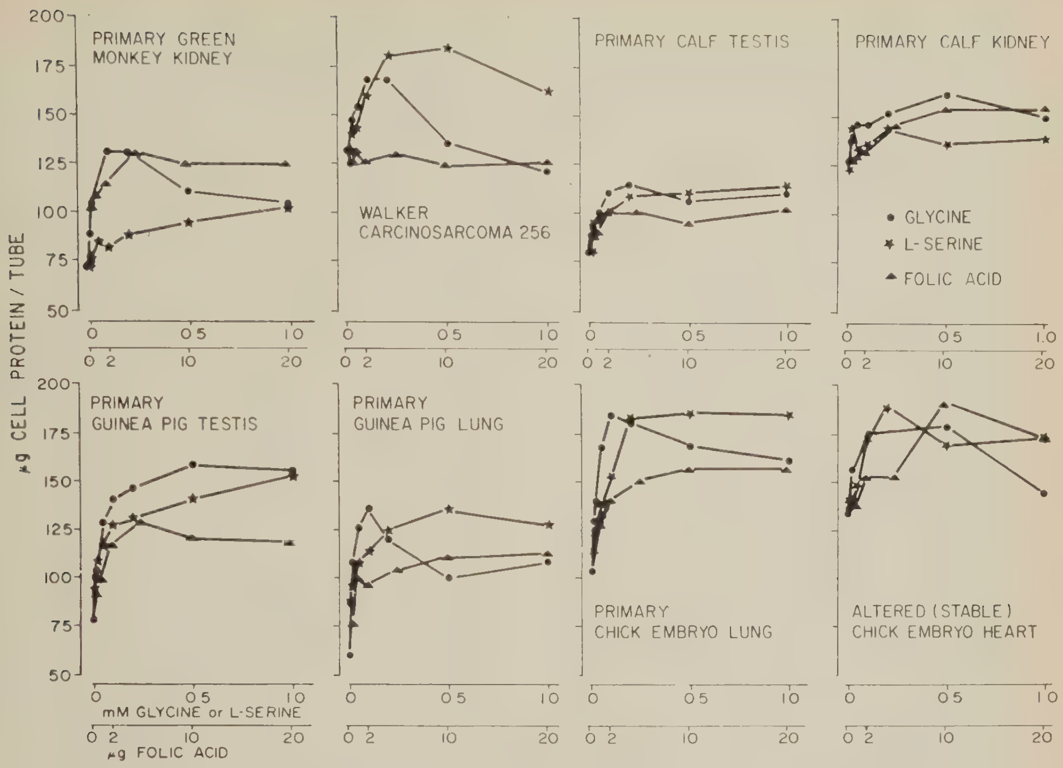

FIG. 1. Growth response of cell cultures to glycine, L-serine, and folic acid.

were particularly responsive to folic and folinic acid, and 2 µg and 0.01 µg respectively of the factors sufficed to promote growth equal to that obtained with glycine.

A more detailed inquiry into the contributions of glycine and L-serine to the cell economy and relation of these amino acids to folic acid in the present tissue culture system appeared desirable. The Walker carcinosarcoma 256 and primary green monkey kidney cells were grown in media which contained 10^{-3} mM amethopterin (4-amino-N^{10}-methylpteroylglutamic acid) and was devoid of folic acid. Initial experiments established that purines, pyrimidines, and glycine were required for growth under these conditions, as shown previously for other cells by Hakala (10,11).

The ability of various purines and derivatives to support growth of cells supplied with glycine and thymidine is shown in Table II. For tumor cells and the primary kidney cells, adenine, adenosine, ATP, hypoxanthine and inosine were effective in approximately equimolar quantities. Responses were determinable at 0.01 mM and maximal at 0.05-0.1 mM. Guanine was only slightly active for primary green monkey kidney. Guanosine and guanylic acid in further experiments were ineffective. Studies with media from which folic acid was omitted but which contained no amethopterin yielded similar results.

Table III shows capacity of several pyrimidines to support growth of the Walker carcinosarcoma and monkey kidney cells in medium containing glycine and adenosine. Both cultures utilized thymidine and thymidylic acid. Thymine was slightly beneficial to the kidney cultures but not to the tumor cells. Cytosine and uracil did not effectively fulfill the pyrimidine requirements. In later experiments 5-methyl-deoxycytidine was shown completely interchangeable with thymidine. These compounds exhibited activity at levels as low as 0.001 mM. Because of this high sensitivity, thymidine requirements could not be demonstrated consistently in media which did not contain amethopterin. Apparently trace quantities of folic acid residual in cells or test medium permitted synthesis of the

TABLE II. Growth Response to Purines by Primary Green Monkey Kidney and Walker Carcinosarcoma 256 Cells in Media Containing Amethopterin, Glycine and Thymidine.

Conc., mM	Adenine	Adenosine	ATP	Hypoxanthine	Inosine	Guanine
			Primary green monkey kidney cells (μg cell protein/tube)			
.0	26	26	26	26	26	26
.005	60	30	30	36	28	
.01	96	64	54	74	60	24
.02	140	110	98	110	120	26
.05	180	172	152	184	166	20
.1	168	192	144	178	156	40
.2	156	176	128	172	162	58
			Walker carcinosarcoma 256 (μg cell protein/tube)			
.0	16	16	16	16	16	16
.01	64	36	44	42	42	18
.02	118	86	66	100	82	16
.05	154	152	136	150	154	16
.1	166	136	140	168	156	16
.2	152	116	80	168	156	12

Basal medium contained .2 mM glycine, .05 mM thymidine, 10^{-3} mM amethopterin. L-serine and folic acid were omitted.

small quantities of thymidine essential to the cells.

Similar experiments to determine the requirement for glycine were conducted in media containing 0.05 mM each of thymidine and adenosine. Growth was negligible at zero levels of glycine, detectable at 0.01 mM, and optimal at 0.1-0.5 mM as observed previously in the glycine stimulation studies in media containing folic acid. However, L-serine was totally ineffective in replacing the glycine. p-aminobenzoic acid did not spare or replace the folic acid requirement of the cultures.

TABLE III. Growth Response to Pyrimidines by Primary Green Monkey Kidney and Walker Carcinosarcoma 256 Cells in Media Containing Amethopterin, Glycine and Adenosine.

Conc., mM	Thymidine	Thymidylic acid	Thymine	Uracil	Cytosine
		Primary green monkey kidney cells (μg cell protein/tube)			
.0	30	30	30	30	30
.001	80	84	36		
.005	156	156	50		
.01	196	198	54	44	46
.02	200	202	68		
.05	202	192	84	40	50
.1	196	198	84	44	
.2	168	176	88	46	44
		Walker carcinosarcoma 256 (μg cell protein/tube)			
.0	10	10	10	10	10
.001	24	26	8		
.005	192	140	8		
.01	174	186	10	12	6
.02	176	172	14		
.05	162	162	14	6	8
.1	152	158	8	6	8
.2	138	138	14		

Basal medium contained .2 mM glycine, .05 mM adenosine and 10^{-3} mM amethopterin. L-serine and folic acid were omitted.

Discussion. Some question may be raised concerning the adequacy of cofactors involved in bio-synthesis of glycine and L-serine. Eagle(12,13) reported the optimal folic acid requirement for stable line cells grown in media devoid of glycine and L-serine to be approximately 0.004-0.04 μg/ml. Haff and Swim reported 0.4 μg/ml to be optimal for repeated subculture of a strain of rabbit fibroblasts(14). Eagle customarily included in his medium 1 μg/ml of each required vitamin (15). Most frequently other media employed in tissue culture have contained 0.1 μg folic acid/ml(16). In the initial studies on glycine and L-serine stimulation described here, folic acid was present at 0.2 μg/ml. Increasing this concentration to 0.5 and 1 μg/ml improved growth to some extent in absence of glycine or L-serine. Nevertheless, maximal stimulation by folic acid generally was not attained until the level reached 5-20 μg/ml. This maximal growth was similarly attained by inclusion of 0.01-5 μg of folinic acid. This active form of folic acid is synthesized from it and is often effective at lower concentrations (17). Fortification of the coenzyme system involved in glycine and L-serine synthesis, therefore, augmented growth of the cultures. However, the enhancement usually was not equivalent to that produced by the amino acids, glycine and L-serine. Evidently synthesis of these compounds remained a limiting factor. The possibility existed that supplementation of the medium with purines and thymidine, other products of folic acid systems, might increase efficiency of glycine synthesis. Addition of these compounds at various levels to the medium in absence of glycine or L-serine did not stimulate as did the amino acids. It may be concluded that glycine and L-serine are desirable constituents of media designed for maximal growth of a wide variety of cell cultures.

Hakala demonstrated replacement of the folic acid requirement of HeLa, sarcoma 180, and J-111 cells by glycine, purines and thymidine(10,11). The work reported here both on the rat Walker carcinosarcoma 256 and primary green monkey kidney cells agreed with her findings in major respects. Adenine and hypoxanthine and their derivatives were effective for the various cell cultures at 0.02-0.1 mM. Guanine was very slightly active. Guanosine and guanylic acid were slightly active for sarcoma 180 while the Walker carcinosarcoma 256 and primary green monkey kidney cell did not respond. Thymidine, thymidylic acid and 5-methyl-deoxycytidine were equally active. As suggested by Hakala, deamination mechanisms for 5-deoxycytidine probably exist in the cultures. In contrast to Walker carcinosarcoma 256, sarcoma 180, and J-111 the green monkey kidney cells were somewhat benefited by thymine. HeLa cells were reported to grow slowly on thymine. In the present work L-serine, unlike glycine did not support cells in media containing amethopterin, thymidine, and adenosine. Omission of L-alanine, pyruvate, alpha-ketoglutarate, and oxalacetate did not depress growth.

As suggested by Lockhart and Eagle(3) leakage of glycine or L-serine may take place from isolated cells in media devoid of glycine or L-serine. Accordingly, growth of isolated cells could fail through loss of compounds which easily escape from the cell and are synthesized at limited rates. Glycine or L-serine has been found in this laboratory to be essential rather than stimulatory for the isolated Walker carcinosarcoma 256 cell. Very small inocula approximating isolated cells may respond to glycine or L-serine as essential amino acids. By contrast, the greater quantities of glycine and L-serine which leak from massive cultures in deficient medium are conserved in utilizable concentrations in the medium. The inocula employed in our studies were at practicable levels which produced sheets of cells within 3-5 days.

Summary. Growth of the majority of a wide variety of primary and stable line cultures was stimulated significantly by inclusion of glycine or L-serine in the medium. Folinic acid or high levels (5-20 μg/ml) of folic acid also accomplished this effect for most cultures, although generally not to the same extent as did amino acids. The requirement of Walker carcinosarcoma 256 and of primary green monkey kidney cells for folic acid was fulfilled by glycine, thymidine, and adenine or several related compounds. L-serine could not replace glycine under similar conditions. The implications of these findings are discussed.

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Diet as Source of Serum Cholesterol in Man.* (25664)

C. BRUCE TAYLOR,† DOROTHY PATTON, NELSON YOGI AND GEORGE E. COX
Dept. of Pathology, Presbyterian-St. Luke's Hospital, Chicago, Ill.

Serum cholesterol in animals and man may be advantageously regarded as having 3 potential sources: (1) synthesis by the liver, (2) synthesis by extrahepatic tissues, and (3) absorption of cholesterol from ingested foods. An inverse relationship between hepatic cholesterol synthesis and absorption of cholesterol from the diet has been demonstrated consistently in several species of laboratory animals. Rapid suppression of hepatic cholesterol synthesis, in response to absorption of dietary cholesterol, is believed to compensate for ingested cholesterol such that total supply of cholesterol (endogenous plus exogenous) to serum tends to remain constant (1). The existence of such a homeostatic mechanism in dogs, rats, and monkeys has been amply demonstrated; its existence in humans has been generally assumed, but never demonstrated. Cholesterol synthesis in extrahepatic tissues generally shows little change in response to dietary cholesterol. The homeostatic mechanism for serum cholesterol therefore appears to be primarily a function of the liver. It would seem, then, that the capacity of this homeostatic mechanism must be close to the maximum capacity of the liver to synthesize cholesterol; that is, the capacity of the liver to compensate for dietary cholesterol by reducing its endogenous synthesis cannot exceed its maximal capacity to synthesize cholesterol. For example, if the liver of man normally has a maximal or potential capacity to synthesize and contribute to serum about 2 g of cholesterol per day, it is reasonable to credit it with a compensation capacity of about 2 g per day. Several workers have suggested 2 g per day as a good approximation of rate of synthesis of serum cholesterol in man (2). No previous study has permitted a decisive interpretation of what tissues participate most prominently in contributing cholesterol to serum of man. Inferring from dogs and rats, it has been widely assumed that most of this 2 g per day is synthesized within the liver. Our study was not designed further to test the validity of the 2 g per day rate, but to determine what proportion of serum cholesterol normally can come from synthesis by liver tissue, and how much from synthesis by extrahepatic tissues. That is, however much cholesterol is synthesized and put into serum daily, whether it be 2 g or more or less, what percentage of this amount originates where?

Method. By feeding a cholesterol-rich diet the liver is suppressed (it is assumed that this suppression phenomenon exists in man as in lower animals) and thereby effectively eliminated as a source of serum cholesterol. If dietary cholesterol is labelled with carbon-14 (4-C-14-cholesterol) the contribution of dietary cholesterol to serum cholesterol can be easily measured by virtue of its radioactivity. Whatever percent of serum cholesterol comes directly from dietary cholesterol, the difference between this and 100% is thought to represent the contribution of extrahepatic tissue. Assuming that the cholesterol-rich diet is replacing the liver as one source of serum cholesterol, then the contribution of the diet should equal that amount that would be the contribution of the liver if the diet contained no cholesterol. In preparation of the diet, 10 g of dry egg yolk solids were taken as equal to 1 egg yolk, with a cholesterol content of 0.25 g. Appropriate amounts of dry egg yolk were suspended in absolute alcohol, radioactive and additional inert cholesterol were dissolved in hot absolute alcohol, and this was then thoroughly mixed into the egg yolk suspension. The mixture was placed in large shallow trays and the alcohol evaporated with the aid of fans. The residue was finely ground, thoroughly mixed, and packed into small souffle cups, 1 dose per cup. This pro-

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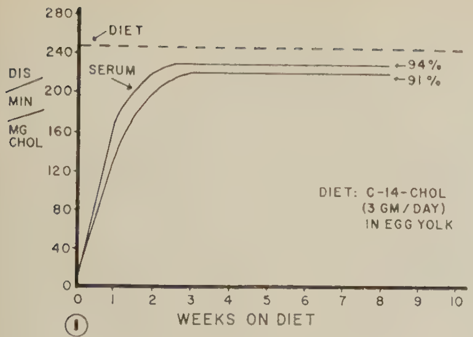
† Present address: Dept. of Pathology, Evanston Hospital, Evanston, Ill.



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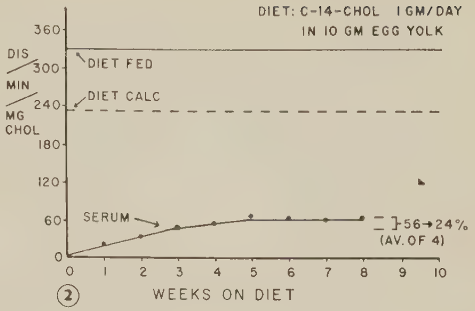

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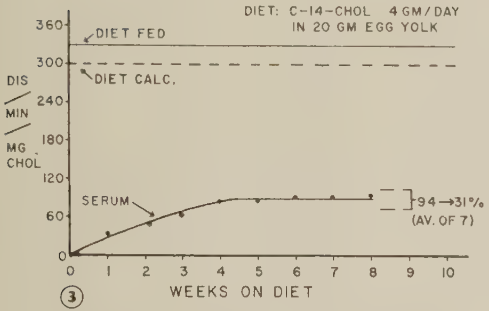

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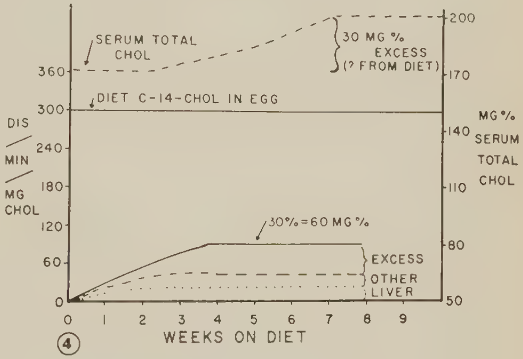

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FIG. 1. Proportion of serum cholesterol derived from diet, in dogs fed a cholesterol-rich diet with C-14-cholesterol mixed in egg yolk.

FIG. 2. Proportion of serum cholesterol derived from diet, in humans receiving a daily total of about 1.4 g cholesterol with 1.0 g supplied as C-14-cholesterol mixed in 10 g egg yolk.

FIG. 3. Proportion of serum cholesterol derived from diet, in humans receiving a daily total of about 4.4 g cholesterol with 4.0 g supplied as C-14-cholesterol mixed in 20 g egg yolk.

FIG. 4. Theoretical considerations in estimating contribution of the liver to serum cholesterol, from data that specifically and directly measures contribution of dietary cholesterol to serum cholesterol.

cedure did not improve the taste quality of the egg yolk, but did facilitate a uniform mixing of the labelled cholesterol with the unlabelled cholesterol including that naturally present in the egg yolk. Volunteer healthy adults ingested this diet for 8 week periods. In Exp. 1, 4 subjects each ingested a daily dose of 10 g egg yolk containing 1 g C-14-cholesterol. In Exp. 2, 7 subjects each ingested a daily dose of 20 g egg yolk containing 4 g C-14-cholesterol. With few exceptions the daily amount was taken in split doses, to facilitate absorption. Blood samples were drawn once weekly, serum cholesterol isolated, washed by the procedure of Sperry and Webb(3), and radioassayed in a Packard Tri-Carb liquid scintillation spectrometer. Aliquots of the diet mixture were also assayed as a check on its composition. Total cholesterol determinations were done by the method of Sperry and Webb.

Results. This study employed a principle previously used by Morris *et al.*(4). This group fed rats a 2% C-14-cholesterol diet and found that 74 to 90% of the serum cholesterol came from dietary cholesterol. The remaining 10 to 26% was believed to represent the contribution of extrahepatic tissues to serum cholesterol in the rat. Applying this principle to dogs, in preliminary studies, we obtained results typified in Fig. 1. The dogs were fed commercial dog biscuits, with daily supplements of 50 g of egg yolk solids containing 3 g cholesterol labelled with a specific activity of 242 DPM/mg (disintegrations per minute per mg of cholesterol). Specific activity of the serum cholesterol quickly rose to peak plateau values of 220 and 228 for the 2 dogs shown. By calculation (specific activity of serum cholesterol ÷ specific activity of dietary cholesterol, × 100) 91 and 94%, respectively, of serum cholesterol came from dietary cholesterol. Presumably, then, 91 and 94% of serum cholesterol would come from the liver if the animals were eating a cholesterol-free diet. These results closely confirm previous studies on dogs by other workers using different methods, all of which demonstrated a remarkably predominant role of the liver in supplying cholesterol to serum in low-cholesterol fed dogs(5).

When the study was begun on human subjects, amount of egg yolk and cholesterol was reduced, because the diet appeared so much less palatable to humans than to the dogs that smaller doses were mandatory. The results from Exp. 1 are shown in Fig. 2. These 4 subjects included 2 females age 22, 1 female age 50 and 1 male age 24 years. Specific activity of their egg yolk ration ("diet fed") was 330 DPM/mg. Because of a calculated average ingestion of nonradioactive cholesterol (in foods) of about 0.4 g per day, overall specific activity of dietary cholesterol ("diet calculated") was estimated to be about 236 DPM/mg. Ingestion of this diet for 8 weeks effected a rise in serum cholesterol radioactivity to a peak plateau value, in one subject, of 60 DPM/mg. The curve in Fig. 2 represents this individual subject, with the points indicating the observed specific activities of serum cholesterol. The 2 horizontal bars at end of curve indicate the range of the 4 cases. Average of the 4 cases is 56 DPM/mg, which demonstrates the diet to be a source of 24% (56 ÷ 236, × 100) of the serum cholesterol. Presumably then the remaining 76% is coming from extrahepatic tissues or residual unsuppressed hepatic cholesterol synthesis.

In Exp. 2 (Fig. 3) the 7 subjects included 5 males and 2 females, ages 20 to 30 years. The 0.4 g nonradioactive cholesterol in their diets was calculated to lower overall dietary cholesterol specific activity from 330 DPM/mg to 300 DPM/mg. A typical 8 week curve, representing actual data of 1 subject, is shown. Horizontal bars at end of curve indicate range of the cases. Average of the 7 cases is 94 DPM/mg, which demonstrates that the diet was supplying 31% of the serum cholesterol in these subjects. The remaining 69% should represent the contribution of extrahepatic tissues only, if hepatic cholesterol synthesis in the human is suppressible by dietary cholesterol.

In both experiments, serum cholesterol specific activity reached its peak value in 4 to 5 weeks, thereafter remaining quite constant to the end of the 8 week study period.

Discussion. If the human liver normally synthesizes cholesterol at a slow rate, then even its complete suppression could offer little compensation for dietary cholesterol. If the human liver synthesizes cholesterol at a rapid rate—but if this rate is not significantly reduced by dietary cholesterol—then again the "compensation capacity" of the liver could not be large. The only available data indicate a very low rate of synthesis, as estimated in liver biopsies surgically removed from patients with non-hepatic diseases(6); however, these patients generally were eating a diet of their choice prior to surgery, thereby leaving open the possibility that the observed low rate might represent a potentially high rate which was being suppressed by dietary cholesterol.

It is not known whether hepatic cholesterol synthesis in man can be markedly suppressed by dietary cholesterol. The technic of comparing uptake of C-14-acetate into serum cholesterol, in subjects on a cholesterol free *vs.* a cholesterol rich diet, shows a striking and unequivocal suppressive effect of cholesterol feeding in dogs(7); however, the same technic, with careful dietary control, showed no suppressive effect of cholesterol feeding in humans(8). This is quite consistent with the observations mentioned above, that human liver normally has a low rate of cholesterol synthesis such that its suppression would not be expected greatly to reduce overall uptake of C-14-acetate into serum cholesterol. Of course, it is also consistent with the alternate, but completely undemonstrated, hypothesis of a high rate which is not reduced by dietary cholesterol.

The data reported here clearly demonstrate a striking difference between cholesterol metabolism of man, and the dog and rat. In dogs and rats on a cholesterol-rich diet, dietary cholesterol can serve as a *major* source of serum cholesterol, supplying between 74 and 94% of the serum cholesterol. In man, dietary cholesterol apparently can supply only about ¼ to ⅓ of the serum cholesterol. Larger daily feedings of egg yolk and cholesterol might increase the percentage of serum cholesterol coming from dietary cholesterol; however the similarity of results of feeding 1 g *vs.* 4 g cholesterol per day, indicates that the picture would not be changed very much by feeding larger amounts of cholesterol. Anyway, if the liver in man is not suppressed by 4 g of cholesterol per day it is unlikely that any reasonable human diet would ever suppress it, and thus its compensation capacity must be quite inefficient at the usual levels of intake of cholesterol.

The actual contribution of liver to serum cholesterol in subjects on a cholesterol-free diet may be much less than the 24 to 31% indicated in Exp. 1 and 2. Two theoretical—unproven but reasonable and likely—considerations are outlined in Fig. 4; note that a scale for mg% serum total cholesterol is superimposed on the right side of this figure. Most of the subjects in Exp. 1 and 2 showed a serum cholesterol elevation of 10 to 40 mg%. Consider a subject whose level increased by 30 mg%, as indicated by the rise from 170 to 200 mg%: it may be reasonably assumed that much or possibly all of this excess came from dietary cholesterol. To facilitate the explanation, assume that all of it came directly from the diet. The diet is then doing more than simply replacing liver as a source of serum cholesterol—it is also supplying an excess of 30 mg%. Therefore, to calculate the potential liver contribution this excess must be subtracted from total dietary contribution. The diet contributed 60 mg% of the serum cholesterol (30% × 200 mg%). Subtracting the 30 mg% excess from the 60 mg% total contribution, leaves 30 mg% (or 15%) as the potential liver contribution. Consider further, that there likely is a little replacement of extrahepatic cholesterol synthesis by dietary cholesterol, such that of this 15%, part very probably represents suppressed extrahepatic cholesterol synthesis. (This part is labelled "other" in Fig. 4.) Subtracting this part from the 15%, leaves only 10 or 5% that may be truly regarded as the contribution of the liver.

The authors wish to emphasize, however, that they do *not* interpret these data to mean that dietary cholesterol is of little importance in hypercholesterolemia in man. The data are interpreted to emphasize the importance of extrahepatic cholesterol synthesis as a normal source of serum cholesterol in man, but not necessarily the source of excess cholesterol in cases of hypercholesterolemia. It is interesting that rats and dogs are both generally regarded as rather resistant to dietary induction of hypercholesterolemia and atherosclerosis—yet the serum in these animals readily obtains its cholesterol from dietary cholesterol, even almost to exclusion of endogenous synthesis as a source of serum cholesterol. The extension of this paradox suggests that in man, dietary cholesterol cannot be ruled out as an important factor in genesis and maintenance of hypercholesterolemia simply because it is not quantitatively the most important source of serum cholesterol.

Summary. Normally active healthy adult volunteers ingested cholesterol-rich diets labelled with 4-C-14-cholesterol. The diets supplied enough cholesterol to suppress hepatic cholesterol synthesis, if the liver is as responsive to dietary cholesterol in man as in lower animals. Radioactivity of serum cholesterol permitted direct calculation of amount of serum cholesterol coming from the diet, which should equal the amount that would come from the liver if the diet supplied none. This amount was found to be 24 to 31%. Presumably, then, at least ¾ of serum cholesterol of these human subjects was derived from cholesterol synthesis in extrahepatic tissues.

The authors are greatly indebted to Joanne Woods, Juana Alvarez, and Hellen Ellis for expert technical assistance.

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Phosphomannose Isomerase Activity in a Spectrum of Normal and Malignant Rat Tissues.* (25665)

Donald E. Kizer and Thomas A. McCoy
Biomedical Division, Samuel Roberts Noble Foundation, Ardmore, Okla.

Mannose catabolism has been observed with slices of Flexner-Jobling carcinoma(1), with Ehrlich ascites cells(2) and with Krebs-2 ascites cells(3). Yushok(3) considered anaerobic mannose catabolism to be mediated by phosphomannose isomerase (PMI) and postulated this enzyme to be rate limiting in mannose catabolism. Recently, hexosamine synthesis from mannose-6-phosphate (M-6-P) and glutamine by Walker carcinosarcoma 256 homogenates was reported(4). It was concluded that the Walker tumor contained PMI or that mannosamine was formed(4). Since these observations indicated entry of mannose into glucose metabolic pathways of malignant tissues, it was important to (a) determine whether tumors possessed PMI, (b) compare PMI activity of malignant tissue to that of normal tissue and (c) determine whether PMI activity was altered during carcinogenesis.

Materials and methods. Transplanted tumors were carried in female Holtzman rats as previously described(5), while primary hepatomas were induced by feeding 0.06% 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) in a semi-synthetic diet described by Medes, *et al.*(6). Since PMI converts M-6-P to fructose-6-phosphate (F-6-P)(7), formation of F-6-P, determined by method of Roe (8), was used to follow extent of reaction. Tissues were homogenized in 0.1 M acetate buffer pH 5.5(9) and centrifuged at 18,000 g for 60 min at 0°C. The supernatant, containing 1-2 mg/ml of protein, was added to a reaction mixture containing 0.03 M-6-P and 0.1 M acetate buffer. Protein concentration was determined by the method of Lowry(10). Aliquots for analyses were removed at appropriate intervals and total incubation period was 10 min at 38°C. A linear relationship between enzymatic activity and time of incubation was obtained. Since an activity maximum was observed at pH 5.4 to 5.5 with both 0.1 M acetate buffer and 0.1 M tris-maleate buffer, the reaction mixture was adjusted to pH 5.5. A unit of enzyme activity is defined as that which produced 0.1 μmole F-6-P/mg protein/10 min. The barium salt of M-6-P[†] was freed of barium by treatment with excess sulfuric acid and, within sensitivity of the Roe method(8), was free of F-6-P.

Results. All normal and malignant tissues studied had PMI activity and the data are summarized in Table I. PMI activity of the Jensen sarcoma and Walker tumor was the same as that of kidney or brain but was significantly higher than the activity of muscle and spleen. Since both of these tumors were grown as intramuscular transplants and moderate contamination with muscle tissue was inevitable, PMI activities reported may be conservative.

Although characteristics of PMI from rabbit muscle(11), brewers yeast(7) and pig erythrocytes(12) were reported, this enzyme from malignant tissues has received limited attention. Thus, purification and inhibition studies were conducted with the PMI from the Walker tumor. Successive fractionations with ammonium sulfate resulted in an approximate 20-fold increase in activity; however, extensive contamination with phosphoglucose isomerase (PGI) persisted. Methanol fractiona-

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* The authors express their appreciation to Boyd Howell for technical assistance.

[†] Nutritional Biochemicals Corporation, Cleveland, O.

TABLE I. Distribution of Phosphomannose Isomerase among Normal Tissues and Transplanted Tumors.

	No. of observations	PMI activity, enzyme units*
Normal tissue		
Kidney	4	2.9 ±.1
Brain	4	2.7 ±.2
Muscle	4	1.9 ±.1
Spleen	4	1.5 ±.1
Transplanted tumor		
Jensen sarcoma	6	2.8 ±.1†
Walker carcinosarcoma 256	6	2.7 ±.1†
Flexner-Jobling carcinoma	6	1.7 ±.2

* Enzyme unit—See text.

† Significantly higher (0.01 P) than value for muscle. Student's "T" test.

tion which was used successfully to separate brewers yeast PMI from PGI(7), yielded high purification of PGI but was ineffective for PMI. Activity was completely inhibited by 5 x 10^{-5} M *p*-chloromercuribenzoate, and activity was partially restored with cysteine. Partial inhibition was observed with *o*-phenanthroline and ethylenediaminotetraacetate. These observations indicated that PMI of the Walker tumor required free sulfhydryl groups and divalent ions for maximum activity. Similar requirements were reported for pig erythrocyte PMI(12).

TABLE II. Phosphomannose Isomerase Activity of Hepatomas and Liver.

Tissue	No. of observations	PMI activity, enzyme units*
Liver	6	1.0 ±.1
Liver adjacent to primary hepatoma	3	.47±.1†
3′-Me-DAB hepatoma‡	6	2.8 ±.4†
Novikoff hepatoma	5	1.4 ±.1
Primary 3′-Me-DAB hepatoma	3	1.4 ±.2

* Enzyme unit—See text.

† Significantly different (0.01 P) than value for liver.

‡ Initially induced with 3′-Me-DAB and carried more than 50 transplant generations.

Comparison of malignant tissue with its normal tissue of origin was achieved by determining PMI activity of normal liver, primary hepatomas and transplanted hepatomas. The data are shown in Table II. Neither the Novikoff hepatoma nor primary lesions induced with 3′-Me-DAB showed PMI activity differing appreciably from PMI activity of normal liver. On the other hand, the transplanted 3′-Me-DAB hepatoma had an activity considerably higher. It would appear that either (a) PMI activity of the original 3′-Me-DAB hepatoma increased during subsequent transplantation or (b) that different hepatomas were induced with the same carcinogen. A similar enzymatic distribution was reported in a study of adenylic acid deaminase activity of primary and transplanted hepatomas(5).

PMI activity of liver tissue adjacent to primary lesions was lowered 50% compared to normal liver. Since microscopic study‡ revealed foci of malignant cells in this tissue, the reductions in PMI activity may have been conservative. These data suggested that PMI activity of normal liver may undergo changes during carcinogenesis and prior to demonstrable neoplasia.

Summary. Homogenates of normal and malignant tissues were centrifuged at 18,000 g and the phosphomannose isomerase (PMI) activity of the supernatants determined. PMI activity of Jensen sarcoma and Walker carcinosarcoma 256 was equivalent to that observed in kidney or brain, but was higher than the activity of muscle. Activities in primary hepatomas, induced with 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) and the Novikoff hepatoma were equivalent to that of normal liver. A transplanted 3′-Me-DAB hepatoma had PMI activity 2-fold higher than normal liver, while activity in liver tissue adjacent to primary hepatomas was approximately half that observed in liver. The enzyme from the Walker tumor was inhibited by *o*-phenanthroline, ethylenediaminotetraacetate and *p*-chloromercuribenzoate (CMB). CMB inhibition was partially reversed by cysteine.

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‡ The authors are grateful to Dr. E. S. Irvine for microscopic examinations.

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Do Mitochondria Participate in General Cardiac Hypertrophy?* (25666)

LOUIS TOBIAN, ODEAN SEVERSEIKE AND JOHN CICH
Dept. of Medicine, University of Minnesota Hospital and School of Medicine, Minneapolis

It is known that muscles which have a constant heavy amount of work, such as the breast muscles of birds have a very high density of mitochondria, while muscles with a lighter work load tend to have fewer mitochondria per unit of muscle mass(1,2). In muscles with a rich supply of mitochondria, it is possible that the increased number of mitochondria is determined by forces at work during embryological development, or that the increased number of mitochondria in these particular muscles is an adaptation to their heavy work load. It therefore seemed of interest to find out how much of an adaptive increase of mitochondrial mass occurs in a typical mammalian muscle which has undergone hypertrophy to meet an increased work load. Our model for muscle hypertrophy was the marked hypertrophy of the wall of the left ventricle which occurs in the rat following production of experimental renal hypertension.

Methods. A number of normal Wistar rats were divided into 2 groups. One group with 12 rats was left intact. This group had blood pressures ranging between 89 and 136 mm Hg. In the second group, one renal artery of each rat was narrowed with a silver clip. Six months later, 14 of the rats with the narrowed renal artery had developed moderate to severe hypertension, as determined by a microphonic manometer(3). Blood pressure was taken on at least 3 separate days within the week prior to killing the rat. The hypertensive rats with blood pressures ranging between 171 and 230 mm Hg were culled out to be compared with the completely normal, intact rats. Arterial pressures averaged 196 for hypertensive rats and 119 for normotensive rats. Our procedure for assessing degree of hypertrophy of the different fractions of the heart was as follows: The animal to be killed was anesthetized with ether and weighed and the heart promptly excised. All the blood was blotted from the cardiac cavities, and the right ventricular wall was trimmed away, as well as both atria, leaving only the left ventricular wall and the interventricular septum. The left ventricle which remained was quickly weighed on a torsion balance, and diced into cubes 1/16th of an inch in size. The cubes were then suspended in .25 molar sucrose containing .01 molar versenate in a Potter-Elvehjem homogenizer. The heart tissue was homogenized for 5 minutes while the homogenizing solution was kept at about 2°C. The homogenate and rinsings were then added to a precooled lusteroid tube, and spun in a Spinco Model L Centrifuge at 600 g for 12 minutes. The supernate from this spinning was transferred to another precooled lusteroid tube. The precipitate was resuspended in .25 molar sucrose and spun for another 15 minutes at 600 g. The supernate from this spinning was added to the other supernate fraction and the precipitate was again resuspended in .25 molar sucrose and the mixture spun at 600 g for one more time. This supernate was then added to the other 2 supernates to comprise the total mitochondrial fraction. The combined supernates containing the mitochondrial

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fraction were then spun in the Spinco Model L Centrifuge at 10,000 g for 20 minutes. The precipitate from this centrifugation was considered to be the genuine mitochondrial fraction. The supernate from this spinning was transferred to another precooled lusteroid tube, then spun for 15 minutes at 105,000 g. The precipitate from this spinning constitutes a sub-mitochondrial fraction which will be discussed below. The supernate from this centrifugation can be considered the "soluble" fraction. This fraction was then added to the original nuclear precipitate leaving 3 separate fractions: 1) the combination of nuclear and soluble fractions; 2) the genuine mitochondrial fraction; and 3) the sub-mitochondrial fraction. Trichloroacetic acid was added to all 3 fractions to provide a final concentration of 10% TCA. The trichloroacetic acid mixtures were shaken for an hour. After all protein had been precipitated, they were spun for 20 minutes at 2,000 rpm in an International Centrifuge. The supernates from all 3 of these fractions were decanted and discarded, and precipitates were heated in an oven at 110°C over night after the method of Hoch and Vallee(4). After heating, the precipitates were allowed to cool in a vacuum desiccator, then weighed on an analytical balance. The weights obtained provide an estimate of the protein content of the various fractions, as outlined in the method of Hoch and Vallee(4). In this system of analysis wet weight of the heart gives an estimate of total heart mass. The fraction which is a combination of the soluble and nuclear fractions gives an estimate of total amount of protein in the heart which is not present in any of the particulate fractions. This fraction will be designated as the "non-particulate fraction." The heated precipitate of the "genuine" mitochondrial fraction gives an estimate of amount of protein in the intact mitochondria. Amount of protein in the sub-mitochondrial fraction was also determined. The particles which make up this fraction are smaller than typical mitochondria. In the usual liver homogenate such particles would be called the "microsomal" fraction. However, heart muscle is said to contain fewer microsomes than liver(5). Ziegler *et al.*(6) have reported studies which suggest that certain sub-mitochondrial particles are primarily composed of fragments of mitochondria. Their evidence reasonably supports this contention. The sub-mitochondrial particles in our fractionation probably represent a mixture of microsomes, mitochondrial fragments and possibly other types of fragments and small particles. Total number of mitochondria in the mitochondrial suspension was determined by counting 4 samples in a Petroff-Hausser counting chamber. Total protein in the mitochondrial fraction was then divided by total number of mitochondria to get average mass of protein per mitochondrion.

Results. As would be expected, average heart weight:body weight ratio among the hypertensive group of rats was 55% greater than that of the normotensive group (Table I). Also, as would be expected, the "non-particulate" proteins of the heart were also increased per unit of body weight in hypertensive group compared to normotensive group. This ratio averaged 53% greater in hypertensives than in normotensives. The mitochondria also participated in the total hypertrophy of the heart. Total mitochondrial protein: body weight ratio was 25% greater in hypertensive rats than in normotensive rats. It is noteworthy that the mitochondrial fraction increased only 25% while the heart as a whole and the "non-particulate" proteins increased about 54% during the hypertrophy, resulting in a significant decrease in amount of mitochondrial protein per unit of left ventricular weight and per unit of "non-particulate" protein. The number of typical mitochondria in the mitochondrial suspension per 100 g of body weight was 50% greater in the hypertensive rats than in the normotensives ($p = .08$). The protein in the sub-mitochondrial particles also underwent a hypertrophy per unit of body weight in the hypertensive rats. This ratio was 37% greater in hypertensive rats than in normotensives. The hypertrophy was not as great, however, as that of the left ventricle as a whole or of the "non-particulate" protein.

The average mass of protein in an individual mitochondrion was somewhat smaller in hypertensive rats than in normotensive ones.

TABLE I. Relationships of Body Weight, Left Ventricular Weight and Mitochondrial and Sub-mitochondrial Proteins in Normal and Hypertensive Rats.

	12 normal rats (N)	14 hypertensive rats (H)	Ratio (H)/(N)	"p" value for difference between (N) and (H)
$\frac{\text{Left ventricular wt}}{\text{Body wt}}$	168	262	1.55/1.00	$< 1 \times 10^{-14}$
$\frac{\text{``Non-particulate'' protein wt}}{\text{Body wt}}$	295	451	1.53/1.00	"
$\frac{\text{Mitochondrial protein wt}}{\text{Body wt}}$	498	622	1.25/1.00	"
$\frac{\text{Total No. of suspended mitochondria}}{\text{100 g body wt}}$	5.67×10^{9}	8.50×10^{9}	1.50/1.00	.08
$\frac{\text{Sub-mitochondrial protein wt}}{\text{Body wt}}$	213	293	1.37/1.00	<.00001
$\frac{\text{Mitochondrial protein wt}}{\text{Left ventricular wt}}$	298	242	.81/1.00	<.00005
$\frac{\text{Mitochondrial protein wt}}{\text{``Non-particulate'' protein wt}}$	169	140	.83/1.00	<.003
$\frac{\text{Sub-mitochondrial protein wt}}{\text{Left ventricular wt}}$	129	114	.88/1.00	.05
$\frac{\text{Sub-mitochondrial protein wt}}{\text{``Non-particulate'' protein wt}}$	73	66	.90/1.00	.13
Avg wt of protein in single mitochondrion (μg)	11.2×10^{-6}	9.0×10^{-6}	.77/1.00	.16

However, the difference was not statistically significant.

Conclusions and summary. 1. Left ventricular weight:body weight ratio of a group of hypertensive rats was 55% greater than that of similar group of normotensive rats. 2. Ratio of "non-particulate" proteins of left ventricle to total body weight was 53% greater in hypertensive group than in normotensive group. 3. Mitochondria of left ventricle also participated in cardiac hypertrophy of the hypertensive group. Mitochondrial protein:body weight ratio was 25% greater in hypertensive group than in normotensive group. 4. Sub-mitochondrial particles of left ventricle, smaller in size than the mitochondria, also participated in cardiac hypertrophy. Ratio of protein in these particles to total body weight was 37% greater in hypertensive series than in normotensive series. 5. Hypertrophy of mitochondria, however, was not as great proportionately as that of left ventricle as a whole. Ratio of mitochondrial protein to total left ventricular weight was 19% less in hypertensive rats than in normotensive group. When expressed as ratio of mitochondrial protein to "non-particulate" protein, the ratio was 17% less in the hypertensive group compared to normotensive group. 6. Hypertrophy of the sub-mitochondrial particles in hypertensive rats was also proportionately less than the hypertrophy of left ventricle as a whole. 7. The mass of protein in individual mitochondrion was slightly less in hypertensive rats than in normotensive group, but the difference was not significant. Total number of mitochondria increased 50% as the left ventricle hypertrophied (p = .08). 8. It seems clear that the total mass of mitochondrial protein in the left ventricle can increase in adaptation to an increase in work load.

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Development of Lipemia in Rats Following Intravenous Injection of India Ink.* (25667)

LAVINA LICKLEY AND FRANK C. MONKHOUSE
Department of Physiology, University of Toronta, Toronto, Canada

In the course of studies of the role of the liver in destruction of clearing factor, Higgins' India Ink was injected into rats. It was observed that blood samples withdrawn one to 2 hours after injection were lactescent. This lactescence was associated with an increase in level of total fatty acids. The lipemia occurred in fasted rats thus indicating that the India Ink was either stimulating an increase in rate of mobilization of fat from the depots or blocking the exit of the mobilized fat from the blood stream. Since the reticuloendothelial system is readily saturated with the India Ink particles, the lipemia could have resulted from inhibition of the normal function of these cells. It has been reported (1) that the reticuloendothelial system functions in uptake of cholesterol from the blood. The evidence(2,3) is against these cells playing any major role in uptake of chylomicrons. The following experiments were carried out in an attempt to determine the mechanism by which India Ink induced lipemia.

Methods and materials. Total fatty acids. The lipids were saponified by heating in presence of alkali and alcohol. The alcohol was removed and the fatty acids were freed by addition of acid in excess. The fatty acids were transferred to a solvent and, after evaporation of the solvent, were titrated by standard alkali. The details were as follows: 0.2 ml of plasma was added to 5 ml of alcohol-ethyl ether mixture (3:1 v/v) and heated to boiling in a water bath. The mixture was then cooled and filtered into a graduated test tube. The residue was reextracted, filtered into the same test tube, and total volume of the extracts made up to 12.0 ml. Five ml of the filtrate was placed in a 15 x 85 mm test tube with screwcap stopper and 0.1 ml of saturated NaOH was added. The mixture was heated in a boiling water bath for 30 min. It was then cooled and while cooling sufficient 33% sulphuric acid solution was added to give an acid reaction. Six ml of petroleum ether was added. The tube was stoppered tightly and shaken vigorously for 1 minute; then 3.5 ml of water was added and the tube shaken again. The mixture was allowed to settle, then two 2 ml aliquots of the clear supernatant were placed in 15 x 85 ml test tubes. The solvent was removed by evaporation and 3 ml of a 0.004% alcohol solution of thymol blue was added. Titration was carried out with 0.02 N KOH. Results are expressed in reference to a standard solution of oleic acid (100 meq/liter).

India Ink. Higgins' India Ink was obtained from the Higgins' Ink Co. Chin Chin India Ink and Pelikan Carbon (C11/1431a) were obtained from the Heinz Jordan and Co. Ltd. These were diluted 3:5 with saline and 1 ml was injected into the exposed jugular vein of each rat. Unless otherwise stated the term India Ink will refer to Higgins' India Ink only.

Rats. The animals used were female rats of the Wistar strain weighing 200-250 g, maintained on a standard chow diet and fasted over-night prior to injection.

Protamine and heparin were supplied by the Connaught Medical Laboratories, Toronto.

Results. In the first experiment India Ink

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* This work was supported by a grant from the Ontario Heart Foundation.

TABLE I. Effect of India Ink on Total Fatty Acids in Plasma of Fasted Rats.

Saline inj.		India Ink inj.	
No. of animals	Fatty acids, mg %	No. of animals	Fatty acids, mg %
32	154 ± 43	37	380 ± 99

was injected into a group of rats and blood samples withdrawn by cardiac puncture at various times after injection. Total fatty acid content of each sample was measured. Lipid levels were increased at 30 minutes and reached a maximum at 60 to 90 minutes after injection. Levels were usually normal by 3 hours after injection. In subsequent experiments, unless otherwise stated, blood samples were taken between 60 and 90 minutes after injection of India Ink.

Table I shows a summary of the results from 70 rats, 32 of which received an injection of saline and 37 an injection of India Ink. There is obviously a highly significant increase in total fatty acids in the animals receiving the India Ink over the saline-injected controls.

Different groups(4,5) have reported that protamine injections cause lipemia in fasted rats. Experiments were carried out to compare development of lipemia following injection of India Ink with that following injection of protamine. For each experiment 16 rats were used, divided into 4 groups of 4 animals each. One group received saline, one group India Ink, one group protamine and the fourth group India Ink and protamine. All solutions were given intravenously *via* the exposed jugular vein, and blood samples taken by cardiac puncture. This experiment was repeated 3 times and the combined results of the 3 experiments are summarized in Fig. 1. Of the 48 rats, 6 died during cardiac puncture and insufficient blood was obtained from them for duplicate measurements. It is obvious from the results that India Ink caused a greater degree of lipemia than protamine and that the 2 together caused a more intense lipemia than either substance alone. This suggests that they caused lipemia by different mechanisms.

It was the opinion of Bragdon and Havel (4) that the protamine-induced lipemia was a result of inhibition of clearing factor. It was of interest, therefore, to determine whether or not heparin injected at the peak of the India Ink-induced lipemia would clear the

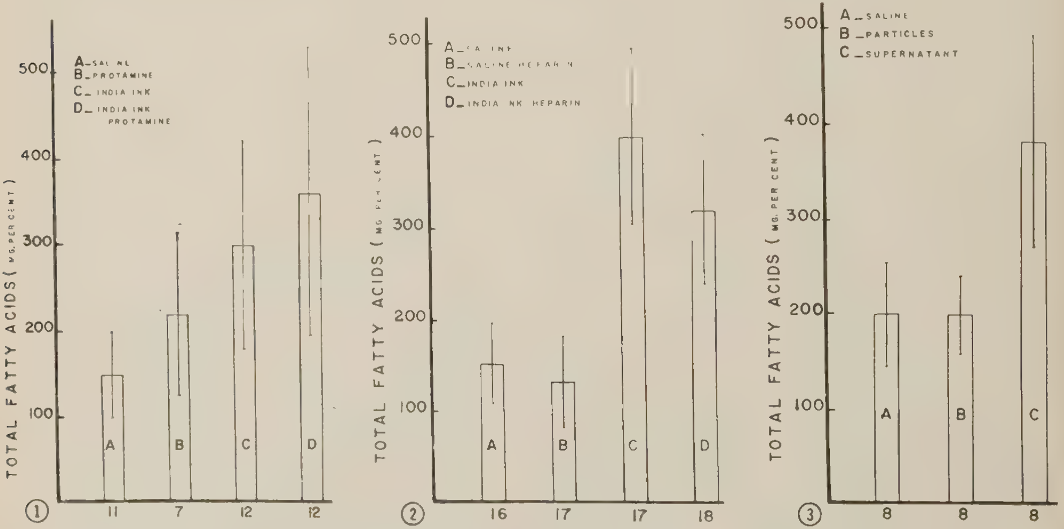

FIG. 1. Total fatty acid level in rat blood following intrav. inj. of various solutions. All samples taken at 60 min. after inj. Mean values and stand. dev. are shown. The number under each column indicates total No. of animals in each group.

FIG. 2. Effect of intrav. heparin on the lipemia caused by inj. of India Ink. Mean values and stand. dev. are shown. Number under each column indicates total No. of animals in each group.

FIG. 3. Comparison of effects of particulate matter and supernatant from India Ink on fatty acid level of blood. Ink was centrifuged at 90,000 g for one hr. Mean values and stand. dev. are given. Number under each column indicates total No. of animals in each group.

plasma and reduce lipemia. Rats were injected with India Ink and 60 minutes later were given 50 units of heparin intravenously. Thirty minutes after heparin injection blood samples were taken. The rats were divided into 4 groups. One group received saline at zero time and 60 minutes later, one group received saline at zero time and heparin 60 minutes later, one group received India Ink at zero time and saline 60 minutes later, and the fourth group received India Ink at zero time and heparin 60 minutes later. Blood samples were taken by cardiac puncture 90 minutes after start of experiment. The combined results of 3 separate experiments are summarized in Fig. 2. The rats which received saline and heparin showed a mean total fatty acid level slightly lower than rats which received only saline. This difference was not statistically significant. Heparin did significantly ($p>0.01$) reduce the lipemia caused by India Ink. This reduction, even after 30 minutes, is far from sufficient to restore the blood level of fatty acids to normal. In many instances the lactescence had disappeared completely. As stated earlier, the lipemia lasted a relatively short time. Microscope examination of liver sections showed that the Kupfer cells were loaded with carbon particles for many hours, even days, after injection. This raised the question whether the lipemia was caused by the carbon particles or by some ingredient or ingredients in the solvent part of the Ink. Pelikan Carbon made up in saline and gelatin(6) did not cause lipemia though it was taken up by the liver as well, if not better, than carbon from India Ink. India Ink was centrifuged in a Spinco high speed centrifuge at 90,000 rpm for 1 hour. The supernatant was pipetted off and the particles of carbon were resuspended in saline. The 2 solutions were injected into rats and the blood level of fatty acids measured one hour later. Results are shown in Fig. 3. It is obvious that the agent responsible for the lipemia was in the supernatant. The president of the Higgins' Ink Co. was kind enough to give us information on materials used in manufacture of their ink. Various combinations of these ingredients were tried. It was found that crude shellac (orange) dissolved in 2% sodium borate caused lipemia in rats of approximately the same degree as the supernatant from the India Ink. Sodium borate alone had no effect on lipid levels.

Discussion. Commercial India Ink has been used(7,8) and continues to be used(9), to saturate and thereby inhibit the phagocytic system within the animal body. Often the conclusions reached from such experiments have disregarded any effects caused by materials in the Ink other than carbon. Halpern *et al.*(6) showed that the shellac in commercial India Ink reacted with certain plasma proteins which, in turn, significantly altered uptake of carbon. Our results show that intravenously injected commercial India Ink can cause a significant increase in fat content of the blood. This lipemia-producing effect also appears to be due to the shellac content of the India Ink. The mechanism by which the shellac induces lipemia has not been determined, but differs from that of protamine. Protamine being an antiheparin inhibits both release of clearing factor(10) and its activity (4). Commercial India Ink does not inhibit release of clearing factor. If anything it prolongs the *in vivo* action of clearing factor by interfering with its destruction. When heparin was injected at the peak of India Ink-induced lipemia, lactescence was much more markedly decreased than total fatty acid level. This difference is greater than when alimentary lipemia is cleared by injection of heparin. It suggests that the India Ink (due to its shellac content) interferes with transport of fat out of the blood. Much more work has yet to be done to be certain of this. Most important to be considered here, we believe, is the demonstration that a lipemia can be produced rapidly by a method hitherto unused. In view of the current emphasis on fat metabolism and its relation to atherosclerosis any addition to the armamentarium for study in this field should be carefully considered.

Summary. Intravenous injection of commercial India Ink in rats caused a significant increase in total fatty acid content of the blood. The effect of a single injection lasted from 2 to 4 hours. The lipemia appeared to be caused by the shellac in the commercial ink. Pelikan Carbon made up in saline and gelatin did not cause lipemia, though it readily saturated the reticular endothelial system. The mechanism by which the shellac induces lipemia is obviously different from that of protamine. India Ink does not prevent *release* of clearing factor (lipoprotein lipase). Heparin injected at peak of the India Ink-induced lipemia reduces the lactescence much more than it reduces total fatty acid level.

We wish to thank Dr. J. Campbell for his advice concerning determination of blood lipids.

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Effects of L-Norepinephrine on Cardiac Metabolism of Dogs in Hemorrhagic Shock.* (25668)

DONALD B. HACKEL

Dept. of Pathology, Western Reserve University School of Medicine, Cleveland Metropolitan General Hospital, O.

Earlier studies in this laboratory have demonstrated a biochemical abnormality of the hearts of dogs in hemorrhagic shock, manifested mainly by a negative extraction of pyruvate by the myocardium. It was also shown that a high per cent of these dogs developed subendocardial hemorrhage and necrosis(1). Since it was hypothesized that these deleterious changes might be partly related to increased secretion of epinephrine during shock, it was felt that detailed studies of the effects of sympathomimetic substances in shock were indicated. We have previously shown that l-norepinephrine does not increase survival rate when given to dogs 10 minutes or longer after production of hemorrhagic hypotension (2), and we have further shown that incidence of myocardial lesions is greater in animals so treated(3). The present report is an extension of the earlier reports, and will be concerned with the effects of l-norepinephrine treatment on some aspects of the myocardial metabolism of dogs in hemorrhagic shock.

Methods. The methods briefly described here have been previously described in detail (1,2). Eighteen mongrel dogs were premedicated with subcutaneous morphine sulfate (3 mg/kg body weight) and anesthetized with equal parts of Dial-urethane[†] and sodium pentobarbital (0.25 ml of this mixture/kg body weight) given intravenously. Each dog was given 200,000 units of penicillin and 0.2 g of streptomycin intramuscularly, and 5 mg/kg of heparin intravenously before the experiment. The aorta, pulmonary artery and coronary sinus were catheterized *via* the femoral artery and jugular veins, respectively, under fluoroscopic control. Systemic arterial and coronary sinus blood samples were analyzed for O_2 and CO_2 (Van Slyke manometric technic), pyruvate(4), lactate(5) and glucose(6). Samples were drawn during the initial control period following which the dogs were bled from the femoral artery over a 10 minute pe-

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[†] The Dial-urethane was generously supplied by CIBA Pharmaceutical Products, Inc., Summit, N. J.

TABLE I. Effects of L-norepinephrine (Series A) or of Blood Infusion (Series B) on Myocardial Metabolism during Shock. (Mean values ± standard error.)

		a. Control	b. Shock	c. Nor-epin. (Series A)	d. Blood (Series B)	Statistical evaluation		
						a–b	b–c	b–d
Pyruv.	Art.	1.43 ± .14	2.47 ± .19	2.48 ± .35	3.95 ± .41	*	0	*
	A–V	.67 ± .11	–.24 ± .15	.18 ± .20	1.01 ± .26	*	0	*
	A–V/A	41.4 ± 6.6	–7.9 ± 6.2	3.2 ± 8.5	27.3 ± 6.2	*	0	*
Lact.	Art.	12.8 ± 1.2	77.9 ± 4.7	77.6 ± 5.9	65.0 ± 12.1	*	0	0
	A–V	6.2 ± .9	5.6 ± 2.1	2.2 ± 2.0	14.0 ± 2.9	0	0	†
	A–V/A	46.5 ± 4.5	9.1 ± 3.0	3.7 ± 3.2	26.2 ± 7.1	*	0	†
Gluc.	Art.	107.0 ± 6.5	306.9 ± 46.4	154.5 ± 49.9	222.0 ± 36.6	*	†	0
	A–V	5.4 ± .6	–3.3 ± 6.5	–.2 ± 8.7	–3.5 ± 2.1	0	0	0
	A–V/A	7.2 ± 1.8	4.6 ± 4.0	7.2 ± 3.9	–2.2 ± 1.5	0	0	0
O_2	Art.	18.1 ± .6	13.6 ± .7	12.2 ± 1.0	18.2 ± 1.1	*	0	*
	C.S.	4.9 ± .5	1.6 ± .3	5.1 ± .9	5.9 ± .8	*	*	*
	A–V/A	73.6 ± 2.2	89.2 ± 1.5	56.0 ± 7.9	67.2 ± 4.3	*	*	*
	Sat'n	91.5 ± 1.2	86.1 ± 2.7	86.2 ± 3.3	93.0 ± 2.3	0	0	0

Statistical evaluation: * Significant difference, with probability <.01 that observed differences could be due to chance. † Significant difference, with probability <.05 and >.01. 0 = No statistically significant difference.

Abbreviations: Art. = Arterial level in mg (or vol) %. A–V = Coronary arteriovenous difference (mg %). A–V/A = Percent extraction (%). C.S. = Coronary sinus level (vol %). Sat'n = Oxygen saturation (%).

riod into a reservoir set to maintain mean systemic blood pressure at 30 mm Hg. After 45 minutes of sustained hypotension, blood samples were again drawn. In one series of 12 dogs (Series A) the cannula between aorta and reservoir was then clamped and an intravenous infusion of l-norepinephrine bitartrate (24 μg/ml) was given at a rate that increased mean arterial blood pressure to 80-90 mm Hg within 5 minutes. Rate of the drip was set to maintain this level of pressure. After 15 minutes the 3rd set of determinations was made, with reservoir blood being reinfused at the same rate that it was withdrawn in the sampling procedure. A second series of 6 dogs (Series B) was studied in this same manner except that, instead of l-norepinephrine, the reservoir blood was infused at a rate that maintained arterial blood pressure at 80-90 mm Hg.

Results. Confirming previous studies from this(1) and other(7) laboratories, a negative myocardial extraction of pyruvate is found during hemorrhagic shock in dogs (Table I). Although extraction (A-V) of lactate is maintained in shock, it is in the face of a markedly increased arterial level, so that the per cent extraction (A-V/A) of lactate during shock is significantly decreased. Infusion of l-norepinephrine does not cause significant change in this picture, but reinfusion of whole blood causes immediate reversion of both pyruvate and lactate extractions toward normal. There is great variation in glucose findings during shock and after treatment with blood or norepinephrine, so that the main change of significance is the increased arterial level of glucose during shock.

These metabolic observations may help to explain our previous finding that l-norepinephrine treatment does not increase per cent survival of dogs in shock(2) and, in fact, results in increased incidence and severity of myocardial damage(3). When hemorrhagic shock is treated by l-norepinephrine without relieving the oligemia, cardiac output is not changed and vascular resistance in the extremities (as determined by paw blood flow) is further increased(2). Since the basic metabolic abnormality is not relieved, the heart is less able to handle the increased work load imposed on it. On the other hand, when blood pressure is increased by blood infusion, cardiac output is returned to the normal range and vascular resistance of the extremities is decreased. Under these circumstances the metabolic pattern of the myocardium reverts to normal.

The reason for the abnormal metabolism of the heart during shock is not known, but it is possible that a toxic product of the peripheral ischemia, transported to the heart, may cause a block in myocardial enzyme systems. Such a "circulating lethal toxin" has been reported by Schweinburg, *et al.*(8).

The observation that l-norepinephrine does not increase incidence of survival in these circumstances has been confirmed by others(9, 10). However, when pressor therapy is instituted very early in the experiment, survival rate appears to be increased(11,12). It is known that cardiovascular responsiveness varies during different stages of shock, so that cardiac output will increase with l-norepinephrine early in shock, but not during later stages(13). Caliva, *et al.*, using polarographic technics, have suggested that the very early treatment of hemorrhagic shock, "because it results in the restoration of oxygen levels, might well prevent or correct metabolic abnormalities"(14).

These findings are compatible with the concept that hypotension by itself, in the absence of excess sympathomimetic activity, is not necessarily deleterious to the myocardium. We have previously shown that marked hypotension produced in human subjects by high spinal anesthesia does not result in evidence of myocardial anoxia or in myocardial metabolic derangements(15). Similarly, Remington, *et al.*, have demonstrated that dibenamine increases survival rate of dogs in hemorrhagic shock(16). Although the bleeding volume is less in the dibenamine-treated dogs, it is apparent that the hypotension alone is not the primary lethal factor. The authors suggest that vasoconstriction, which is presumably prevented by the adrenergic blocking agent, is a precipitating factor in irreversibility.

Summary and conclusions. The myocardial metabolism of 18 intact dogs was studied during an initial control period and after production of hemorrhagic shock. Some of the dogs were subsequently treated with l-norepinephrine and some with blood. An abnormal myocardial metabolic pattern was demonstrated during shock which was relieved by blood infusion but not by l-norepinephrine. It is suggested that the reason that l-norepinephrine treatment does not increase survival rate of dogs in shock is because it does not correct the basic metabolic abnormality and at the same time imposes a heavier burden of work on the heart.

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Effects of Lipoid A Component of *Escherichia coli* Endotoxin on Dermal Reactivity of Rabbits to Epinephrine.* (25669)

ERWIN NETER, HIROSHI ANZAI, EUGENE A. GORZYNSKI, ALOIS NOWOTNY,
AND OTTO WESTPHAL
*Children's Hospital, Depts. of Bacteriology and Pediatrics, University of Buffalo Medical School,
Buffalo, N. Y., and Wander-Forschungsinst., Freiburg, Germany*

The endotoxic complex of Gram-negative bacilli is composed of polysaccharide, lipoid, and protein. Two lipoid components, A and B, have been isolated(1). The latter is easily extractable from the endotoxin by fat solvents. In contrast, lipoid A is firmly bound to polysaccharide (lipopolysaccharide). In experimental animals the endotoxic complexes and the purified lipopolysaccharides derived therefrom produce the well known nonspecific effects, such as fever, Shwartzman reaction etc.(2-6). It is the polysaccharide moiety of the complex which is responsible for serologic specificity. Recent studies(7) strongly suggest that toxicity and pyrogenicity of the endotoxins are due to the lipoid A component. Evidence for this conclusion was obtained by demonstration that artificial complexes made up of lipoid A and an inert carrier, such as casein, exert these biologic effects. The inert carrier merely renders water-soluble the otherwise poorly soluble lipoid A, a function which in the natural endotoxic lipopolysaccharide complex is performed by the polysaccharide moiety. Westphal *et al.*(7) isolated from *E. coli* endotoxin the lipoid A component in aqueous solution of varying degrees of dispersion. The acute endotoxic activity of lipoid A depends largely upon degree of dispersion, being maximal in the original endotoxic complex. Lipoid A also markedly enhances nonspecific resistance of mice to experimental infection(7). The present experiments revealed that purified lipoid A, like the lipopolysaccharide complex, strikingly alters dermal reactivity of rabbits to epinephrine.

Materials and methods. Highly purified lipopolysaccharide from *E. coli* 0111:B4 was prepared according to the method described previously(8). Lipoid A was isolated from the lipopolysaccharide by hydrolysis and further purified by fractionation with organic solvents. The purified product, a white powder with a melting point of 180-82°C, was free of polysaccharide constituents. It contained 19% of glucosamine(1) and gave the following analytical figures: C 61.1, H 9.5, N 2.7, P 1.9, (C)-CH_3 4.1%, respectively. A solution of lipoid A in pyridine was added to an equal volume of 0.5% low molecular dextran. The mixture was concentrated under reduced pressure until completely free of pyridine and enough water was added to give a final concentration of 1000 μg/ml of lipoid A in 0.5% dextran. This solution was kept at 4°C. Immediately prior to injection into rabbits, the appropriate dilution was prepared aseptically in phosphate buffer (pH 7.3; Difco). The dextran diluent and the lipopolysaccharide, from which lipoid A was prepared, were used in parallel experiments. Lipoid A, lipopolysaccharide, or dextran diluent, in appropriate dilutions, was injected intravenously into white female rabbits, weighing between 1.7 and 2.3 kg. Within a few minutes thereafter epinephrine (in aqueous solution or peanut oil) as well as saline solution, in amounts of 0.1 ml, were injected into the shaved abdominal skin at different sites. At intervals for several days, the dermal lesions were measured and graded according to intensity as strongly positive, weakly positive, or negative; presence or absence of hemorrhages was recorded.

Results. The alteration of dermal reactivity to epinephrine effected by intravenously injected lipoid A, lipopolysaccharide, and 0.5% dextran is shown in Table I.

Of 71 rabbits which received from 2 to 50 μg/kg of lipoid A intravenously, 26 gave strong reactions at site of epinephrine in water (100 μg) and 40 animals at that of epinephrine in oil (200 μg). Lipoid A in effective doses caused strongly positive reactions to both aqueous epinephrine and epinephrine in

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TABLE I. Effects of *E. coli* Lipoid A and Lipopolysaccharide on Dermal Reactivity of Rabbits to Epinephrine.

	Epinephrine (100 μg)			Epinephrine in oil (200 μg)			
Materials and dosage	Strongly positive	Weakly positive	Negative	Strongly positive	Weakly positive	Negative	Total
Lipoid A							
50 μg/kg	2	2	4	6	1	1	8
20	19	10	8	22	10	5	37
5	1	3	10	5	4	5	14
2	4	8	0	7	5	0	12
.5	0	0	4	0	1	3	4
.2	0	0	8	1	2	5	8
Lipopolysaccharide							
2 μg/kg	3	1	0	3	0	1	4
.2	3	2	0	5	0	0	5
.05	0	0	2	1	1	0	2
.02	0	0	6	0	0	6	6
Diluent for Lipoid A							
1:20*	0	2	2	0	0	4	4
1:50	0	3	12	0	4	11	15
1:200	0	0	5	0	0	5	5
1:200	†	†	†	0	0	3	3

* Equivalent to 50 μg/kg of Lipoid A. † Not done.

oil in many animals. A few rabbits, however, gave such reactions to only one or the other. None of the 27 animals injected with dextran had strongly positive reactions. Numerous other rabbits injected intravenously with phosphate buffer, used as diluent, (not shown in Table) failed to react to epinephrine, indicating that endotoxic lipopolysaccharide was not encountered as a contaminant. Weakly positive reactions were seen in rabbits injected with the dextran diluent in amounts equivalent to 20 and 50 μg/kg of lipoid A, but not in amounts equivalent to 5 μg/kg. Therefore, weakly positive reactions obtained in 11 rabbits that were given 5 μg/kg of lipoid A or less, appear to be significant. Epinephrine in amounts of 10 and 1 μg failed to elicit the reaction in rabbits injected with lipoid A in effective doses. It is evident, therefore, that lipoid A obtained from *E. coli* 0111:B4 markedly alters dermal reactivity of rabbits to epinephrine.

The original lipopolysaccharide, from which the lipoid A fraction was obtained, similarly alters reactivity to epinephrine. Statistical analysis (courtesy of Miss V. Pessin) revealed that the lipopolysaccharide is approximately 10 times more effective than lipoid A, a finding that agrees with the observation on relative pyrogenicity of these compounds(7).

On gross examination the epinephrine lesions following intravenous injection of lipoid A (20 and 50 μg/kg) were red or brown in approximately 25% of the animals and hemorrhagic in 75%. Typical reactions are shown in Fig. 1. The mean size was 16 x 53 mm, with a range of 10 to 30 x 20 to 90 mm. No difference was noted in appearance of reactions between aqueous epinephrine and epinephrine in oil. Hemorrhages were seen less frequently in animals receiving smaller amounts of lipoid A.

The question arises as to whether the epinephrine lesion is due to localization of intravenously administered lipoid A in the skin injected with epinephrine. To test this hypothesis, 5 rabbits were injected intravenously with lipoid A (20 μg/kg) and intracutaneously at different sites with epinephrine, lipoid A (2 μg/kg), and saline. Lesions did not develop at site of intradermally administered lipoid A.

The possibility was considered that shaving may contribute to development of the epinephrine reaction. Several rabbits were injected with lipoid A intravenously and with epinephrine into the unshaved abdominal skin. On the following day, when the results were determined, the skin was shaved. Marked reactions were present. These findings indicate that shaving is not the determinant factor.

Five rabbits were injected intravenously

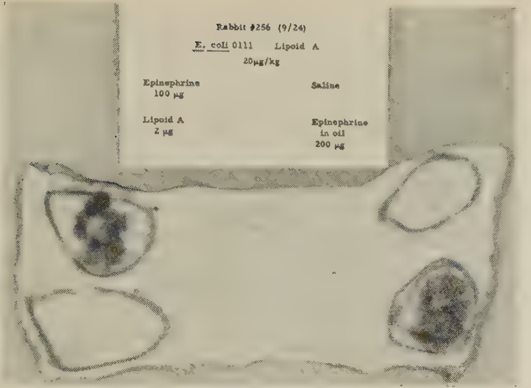

FIG. 1. Effect of lipoid A on dermal reactivity to epinephrine.

with 0.5 mg/kg of the adrenergic blocking agent phenoxybenzamine hydrochloride (Dibenzyline), and 5 control rabbits with saline; 6 hours later all animals received lipoid A (20 µg/kg) intravenously and epinephrine intradermally. No reaction whatever occurred in the former animals, whereas 4 out of 5 control rabbits showed marked lesions. It is concluded, therefore, that phenoxybenzamine hydrochloride inhibits dermal reactivity to epinephrine in rabbits injected with lipoid A.

Discussion. The present investigation has revealed that the lipoid A component of endotoxin obtained from *E. coli* 0111:B4, upon intravenous injection, alters dermal reactivity of rabbits to epinephrine. The lipoid A component is active in amounts as small as 2 to 5 µg/kg. Its potency appears to be approximately one-tenth of that of the original lipopolysaccharide. A similar 10-fold difference in pyrogenic potency in horses was observed with the same preparations(7). The observation on alteration of dermal reactivity of rabbits to epinephrine by intravenously injected endotoxin confirms the findings of Thomas (9). The question remains as to whether chemical compounds of the endotoxic complex other than lipoid A are responsible for some of the numerous nonspecific biologic effects, and/or whether the other components of the complex, such as the polysaccharide, merely affect physico-chemical characteristics of lipoid A. The difference in activity between lipoid A and lipopolysaccharide may be due to differences in degree of dispersion of lipoid A component or chemical alterations (hydrolysis etc.) taking place during preparation of the latter. It is conceivable that in the natural lipopolysaccharide complex lipoid A is better dispersed than in the preparation dissolved in dextran. The findings reported here further support the thesis that at least some of the toxic effects of endotoxins from Gram-negative bacilli are mainly due to lipoid A component. Recently, Ribi and co-workers isolated an aqueous ether extract from *Salmonella enteritidis* which had the biologic properties of a Boivin-type antigen, yet had an exceptionally low lipoid content(10).

The pyrogenic effect of lipoid A and of lipopolysaccharide is abolished by horse serum (11,12). This inactivation is not due to antibody. It remains to be ascertained whether serum also inhibits the effects of lipoid A on reactivity of rabbits to epinephrine. Zweifach and associates(13) observed that endotoxin alters reactivity to epinephrine of vessels of the isolated rabbit ear and of the mesoappendix of the rat. On the other hand, Meyer and Ballin(14) found only slight potentiation of epinephrine activity after small doses of endotoxin and then only if barbiturate, but not ether anesthesia, was employed. The question arises as to whether endotoxin and lipoid A affect different vascular systems differently. For example, in our own laboratories we have failed to elicit the epinephrine reaction in the ear of the rabbit following intravenous injection of a potent staphylococcal toxin or of *E. coli* lipopolysaccharide, although these preparations were highly effective in producing the epinephrine lesion in the skin of the abdomen and back.

Of great interest are the observations of Gatling(15), that epinephrine elicits a striking local reaction in hypersensitive rabbits, provided both antigen and antibody are present in the circulation. It will be of interest to determine whether pre-treatment of animals with lipoid A alters this reactivity. The possibility may be considered that the remarkable reactivity of animals and man to minute amounts of endotoxins may be related to previous exposure, under natural conditions, to materials containing lipoid A, resulting in altered reactivity of certain tissues to endotoxins and their lipoid A component.

Summary. The purified and polysaccharide-free lipoid A component of *E. coli* 0111:B4 endotoxin, upon intravenous injection into rabbits, strikingly alters dermal reactivity to epinephrine. Grossly, the lesions thus produced are red or brownish in appearance or show marked hemorrhages. Lipoid A is active in amounts as small as 2 μg/kg. The original lipopolysaccharide is approximately 10 times more potent than the lipoid A fraction obtained therefrom. Phenoxybenzamine hydrochloride inhibits the epinephrine reaction in rabbits injected with lipoid A.

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Accumulation of Dermal Mucopolysaccharides in Animals Following Injection of Estradiol Benzoate.* (25670)

GEORGE H. WARREN AND RAYMOND FAGAN
Wyeth Inst. for Medical Research, Radnor, Pa.

It has been demonstrated that following administration of estrogenic hormones the amount of ground substance mucopolysaccharides can be increased in immature monkeys of both sexes(1,2). Similar effects were obtained by estradiol following local application of the hormone on skin of rhino and hairless mice(3), and in mice injected subcutaneously with this hormone(4). That part of this increase in dermal ground substance is due to hyaluronic acid or chondroitin sulfate was demonstrated by Schmidt(5). This paper presents the effect of a single subcutaneous inoculation of estradiol benzoate on dermal mucopolysaccharides of mice, rats, guinea pigs and hamsters.

Materials and methods. Male and female white Swiss mice weighing 12 to 15 g and 20 to 22 g respectively, female albino rats weighing 60 to 75 g, female Hartley line guinea pigs weighing 225 to 275 g and female Golden hamsters weighing 60 to 75 g were studied. Animals were obtained from local dealers. Mice, rats and hamsters were fed Purina lab chow while guinea pigs were fed Purina rabbit chow and lettuce *ad lib.* Animals had free access to water. Test animals received a single subcutaneous injection of 0.1 ml of a suspension of estradiol benzoate in sesame oil in inguinal region. Control animals received 0.1 ml of sesame oil. At 2 day intervals beginning with zero days and usually extending through the 21st, duplicate animals were sacrificed; mice by severing cervical cord, other animals by ether. Fur was initially removed

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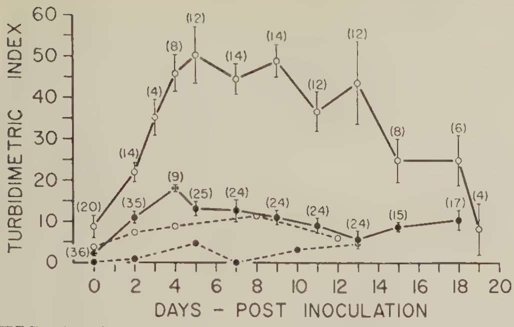

FIG. 1. Accumulation of mucopolysaccharides in skin of mice inoculated with 100 μg estradiol benzoate in sesame oil. Legend: No. in parenthesis is No. of mice used to determine point. Vertical lines indicate plus and minus one stand. error of mean which determines point. ●- - -● Abdomen control. ○- - -○ Back control. ●——● Abdomen treated. ○——○ Back treated.

with clipper. To remove remaining stubble depilatory was applied. After removing excess depilatory with tap water, pieces of skin were removed and placed in hot air oven (60°C) overnight. The effect of estradiol benzoate on accumulation of mucopolysaccharides in skin of test animals was assayed by 2 different methods: (A) turbidimetric assay employed for titration of hyaluronic acid and (B) chemical determination of hexosamine. For turbidimetric determinations samples were removed from oven the following day, weighed, ground in mortar and pestle and suspended in 10 ml of 0.85% saline solution. This suspension was then kept at 5°C overnight. After centrifugation at 1500 rpm for 5 minutes, the supernatant fluid was tested for mucopolysaccharides which are attacked by hyaluronidase in accordance with turbidimetric method described previously(6). An index of mucopolysaccharide accumulation was calculated by taking the difference between optical density of an aliquot of solution before and after depolymerization with 150 USP units of bovine testicular hyaluronidase, then dividing this value by weight of sample in mg and multiplying quotient by 100. For determination of hexosamine in skin samples, the procedure was similar through the drying stage. Thereafter, oils were removed by cutting skin samples into narrow strips and shaking them in acetone on shaking machine for 30 minutes. Three to 4 washings were usually sufficient. Samples were then treated by the method of Rimington(7) with the modification of protein hydrolysis maintained for 24 hours using 3N HCl as opposed to 4 hours using N HCl.

Results. Turbidimetric determinations for mucopolysaccharides which are depolymerized by hyaluronidase and hexosamine assays revealed that evidence of increased ground substance accumulation could be demonstrated in dorsal skin of the mouse only (Fig. 1, Tables I, II). A slight increase of ground substance was noted in abdominal skin to fourth day after inoculation. However, values fell almost to control levels subsequently (Fig. 1). Since the mouse appeared unique with respect to increased ground substance accumulation, it was necessary to determine whether method of sacrifice was responsible for disparity in response. Therefore, several mice as well as other species were sacrificed with ether. Turbidimetric analysis revealed no differences between mice sacrificed with ether or by severing the cervical cord.

An arbitrary standard was derived from Fig. 1 to evaluate other variables. Since peak of estradiol activity occurred from 4th through 13th day post-inoculation, values for 4th to 13th day were averaged. This value was used as index to measure effect of variables.

Data summarized in Table II demonstrate

TABLE I. Glucosamine in Skin of Mice Inoculated Subcutaneously with Estradiol Benzoate in Sesame Oil.

Age of mice (wk)	Estradiol benzoate (μg)	Skin from	Glucosamine, mg/100 g* Time after inoc. (days) 0	2	5	7
3	100	Abdomen	313	481	484	674
3	1000		397	447	551	632
3	Control		388	558	455	514
3	100	Back	391	663	527	1054
3	1000		432	614	788	942
3	Control		316	520	422	380
8	100	Abdomen	330	388	499	488
8	1000		415	318	489	496
8	Control		451	421	385	543
8	100	Back	312	495	745	754
8	1000		509	543	720	699
8	Control		406	357	405	548

* Each value represents mean of samples from each of 2 mice.

TABLE II. Mucopolysaccharides in Skins of Animals after Subcutaneous Inoculation of Estradiol Benzoate in Sesame Oil.

Animal	Sex	Age or wt	Skin from	Drug and/or procedure	Dose	Avg turbidity index of ground substance from 4-13 day*	No. of animals	Range of individual determinations
Mouse	♂	3 wk	Dorsum	Estradiol	10 μg	35.5	10	9.2– 11.2
	♂	8			”	33.0	”	4.8– 60.5
	♂	3			100	50.1	”	8.0– 62.0
	♂	8			”	33.5	18	6.9– 54.5
	♂	3			”			
				Estradiol + cortisone	4.8 mg	42.8	8	17.4– 62.5
	♂	3		Cortisone	”	10.0	8	4.5– 14.3
	♂	3		Castration + estradiol	100 μg	39.8	8	13.6– 63.7
	♀	3		Estradiol	”	63.4	4	22.4– 90.9
	♀	8			”	51.4	40	18.5–104.2
	♀	3			1000	40.8	4	22.2– 61.4
	♀	8			”	54.0	4	29.2– 91.9
	♀	3		Sesame oil	.1 ml	6.8	4	3.9– 10.9
	♀	8			”	6.2	8	3.7– 17.8
Rat	♀	60– 75 g	Abdomen	Estradiol	100 μg	9.6	10	2.7– 13.3
	♀	60– 75	Dorsum		”	9.8	”	1.0– 14.9
	♀	60– 75	Abdomen		1000	10.1	12	3.2– 21.5
	♀	60– 75	Dorsum		”	10.9	”	1.6– 18.0
Guinea pig	♀	225–275 g	Abdomen		”	1.6	10	.0– 4.5
	♀	225–275	Dorsum		”	4.8	”	2.7– 8.3
Hamster	♀	60– 75 g	Abdomen		”	6.0	”	.0– 15.0
	♀	60– 75	Dorsum		”	3.2	”	.0– 6.8

* Since deposition of ground substance shows a plateau between 4th and 13th day (Fig. 1), in order to compare effect of different variables, avg value of turbidity index within these 2 time intervals was chosen.

that response of the mouse to estradiol is independent of age, sex or dose. Three and 8-week-old mice responded similarly to estradiol benzoate. In addition when varying doses of estradiol benzoate were inoculated *i.e.*, 10 μg, 100 μg, and 1000 μg, no significant differences were found in amount of increased dermal ground substance or in length of time excess material remained. Males as well as females responded the same way as far as dermal ground substance was concerned. Furthermore, male mice castrated at 3 to 4 weeks of age did not appear to differ in response from normal male or females.

In other experiments, after having received estradiol benzoate, 3-week-old male mice were inoculated *I.M.* with 1.2 mg of cortisone acetate every other day for a week for a total of 4.8 mg. Despite this regimen, no reversal of ground substance accumulation could be demonstrated (Table II).

Discussion. These data suggest that when estradiol benzoate is inoculated into rodents such as mice, rats, guinea pigs and hamsters in doses previously described, only the mouse responds with accumulation of excess dermal ground substance. If other animals responded to inoculation it was not with any substance detectable by our assay methods. Furthermore, the response seemed confined usually to skin of dorsal region. Although accumulation of ground substance occurred exclusively in the mouse, wide variation in response was noted (Table II). It was thought that estrous cycles in the mice might account in part for this variation. To elucidate this one possible controlling mechanism, random samples of female mice were autopsied and gross examinations of uteri were made. However, no consistent correlations between engorged or normal uterus and degree of activity of estradiol in effecting accumulation of ground substance could be made.

The amount of ground substance mucopolysaccharide in skin can be the steady state of 2 opposing rates: rate of synthesis and rate of depolymerization. Whether estradiol exerts its effect by increasing rate of synthesis while rate of depolymerization remains the same or whether accumulation results from slowing of rate of depolymerization while rate of synthesis remains constant is an interesting speculation on which we have no evidence.

Although we did not succeed in modifying the effects of estradiol on mucopolysaccharides we confirmed an observation of Schmidt's(5) that cortisone had no effect on accumulation of mucopolysaccharides in mouse skin.

Since some tumors(8-10) contain large amounts of hyaluronic acid, it was of interest to study the effects of estradiol on their growth *in vivo* and *in vitro*. Preliminary studies were initiated to determine whether the accumulated ground substance could act to retard or at least contain growth of these tumors. Studies have also been started to determine whether the accumulated mucopolysaccharide might also cause local accumulation of antibodies in skin and hence act as a deterrent to local infection.

Summary. Accumulation of mucopolysaccharides in response to estradiol benzoate inoculation in the skin of various rodents was studied. Only white Swiss mice responded with increased accumulation. Rats, guinea pigs, and hamsters did not show this response. Neither age, sex of the animal, nor dose of estradiol had any effect on amount of mucopolysaccharide accumulated. This activity of estradiol was neither effected by castration of males nor reversed by administration of cortisone.

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Blood Glucose Distribution in the Domestic Fowl. (25671)

DANIEL N. TAPPER AND MORLEY R. KARE
Dept. of Physiology, N. Y. S. Vet. College, Cornell University, Ithaca

It has been demonstrated(1) that in adult laying hens the circulating blood glucose is transported almost exclusively in the plasma. In this respect these birds are similar to the adults of many subprimate mammals(2). Blood glucose has been found to be almost equally distributed in the blood of the fetus and of the newborn in swine, sheep and cattle (2,3). A gradual shift in distribution occurs within the first few months of life at which time almost all of the sugar is in the plasma. Plasma of the newborn foal contains essentially all of the glucose at birth(2). It has been suggested that the changes in swine and ruminants are associated with the change-over from fetal to adult erythrocytes and differences in glucose metabolism observed between the 2 types of red cells(4,5). Foal blood contains only adult cells at birth. In the chicken, replacement of fetal erythrocytes by adult types is said to be completed by the third week after hatching(6). This study deals with distribution of circulating glucose in the blood of the chicken from time of hatching until maturity.

Materials and methods. Eighty-five chickens were used. With the exception of 24 Rhode Island Red chicks, ages 2, 3 and 4

TABLE I. Distribution of Blood Glucose in the Chicken.

Group	No.	Packed-cell vol, %	Whole blood glucose, mg %	Plasma glucose, mg %	Calculated plasma glucose, mg %	% glucose in plasma
Newly-hatched S.C.W.L., ♂-♀	24	26.5 ± .69*	167 ± 4.3	235 ± 4.5	227 ± 4.4	103.8
2-wk-old R.I.R., ♂-♀	8	28.8 ± .64	162 ± 6.8	226 ± 8.5	227 ± 6.4	99.3
3-wk-old R.I.R., ♂-♀	8	28.8 ± .62	173 ± 3.2	222 ± 4.0	242 ± 3.2	92.0
4-wk-old R.I.R., ♂-♀	8	30.5 ± .86	184 ± 5.0	259 ± 9.4	265 ± 5.3	98.0
7-wk-old S.C.W.L., ♀	7	31.9 ± .50	156 ± 2.8	224 ± 3.3	229 ± 3.8	97.6
4½-mo-old S.C.W.L., ♂	6	32.3 ±1.39	169 ± 7.4	242 ±10.5	250 ± 8.2	96.7
6½-mo-old S.C.W.L., ♂	6	37.3 ± .84	188 ± 2.2	273 ± 4.6	300 ± 3.5	91.0
1-yr-old S.C.W.L., ♂	6	42.7 ±1.18	164 ± 2.4	262 ± 5.4	287 ± 3.7	91.5
Adult laying hens, S.C.W.L.	12	29.4 ± .63	173 ± 3.0	233 ± 4.4	245 ± 4.1	95.4

* Mean and stand. dev. of mean.

weeks, the birds were single comb White Leghorns. Ages and sexes are given in Table I. The newly-hatched chicks were not fasted; 2, 3, 4 and 7 week-old birds were fasted for 12 hours; 4½ month, 6½ month and 1 year old males were fasted for 12, 16 and 18 hours, respectively, and adult laying hens were fasted for 18 hours. Blood was taken by heart puncture with a heparin-wetted syringe. Packed-cell volumes were determined by the high-force micro-capillary method. Blood and plasma glucoses were measured by the Somogyi iodometric titration method on from 1:10 to 1:80 filtrate dilutions depending on quantity of blood available. The parameter, calculated plasma glucose, was computed using measured blood glucose and packed-cell-volume values.

Results and conclusions. The results of these experiments are shown in Table I. Blood glucose was distributed almost entirely in the plasma at all ages studied. Erythrocytes of the newly-hatched chick do not have more glucose in them than do those of the adult bird. Quantity of glucose found in the red cells of the newly-hatched and young chicks is less than that reported for neonatal and young animals of other species. Replacement of fetal red cells by adult types which takes place in the first 3 weeks posthatching is not characterized by a glucose distribution shift. The partition at hatching is similar to that in the foal. Since the foal has adult erythrocytes in the blood at birth and the chick has a mixture of adult and fetal cells, it would appear that the differences in distribution of blood sugar as observed in other species may not be attributed simply to differences in carbohydrate metabolism of the 2 cell types.

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Some Differences Among Various Mammalian Cell Lines in Reversal of Growth Inhibition by 5-Fluorouracil.*† (25672)

MARVIN A. RICH, LEONARD SASLAW‡ AND MAXWELL L. EIDINOFF
(Introduced by D. A. Clarke)
Division of Biophysics, Sloan-Kettering Inst., and Sloan-Kettering Division of Cornell University Medical College, N. Y.

Growth inhibition of H. Ep. #1 cells(1) in culture by 5-fluorouracil and 5-fluorodeoxyuridine and attempts to reverse this inhibition by addition of related metabolites has been reported(2,3). Thymidine completely reversed growth inhibition by 5-fluorodeoxyuridine while thymine had relatively little effect. Furthermore, inhibition of growth by 5-fluorouracil could not be reversed by either thymine or thymidine. In HeLa cells in culture, Ackermann *et al.* reported that thymine as well as thymidine reversed growth inhibition by 5-fluorouracil(4). Both of these cell lines are derived from a human cervical carcinoma. Consequently, toxicity and reversal studies were carried out for both cell lines under comparable conditions. Experiments were also carried out using several other cell lines. Reversal studies demonstrated differences among cell lines with respect to reversal of growth inhibition by 5-fluorouracil and 5-fluorodeoxyuridine.

Materials and methods. The cell lines studied were: H. Ep. #1(1) and HeLa(5), derived from human adult epidermoid carcinomas of the cervix; H. Ep. #2(1), derived from adult human epidermoid carcinoma of the larynx; Chang's conjunctiva(6), derived from normal adult, human conjunctiva; AMK(7) derived from normal, adult monkey kidney; V-1 and V-2(8), derived from normal, adult Chinese hamster lung; and L strain(9) derived from normal, adult mouse connective tissue. Stock cultures of H. Ep. #1, HeLa, AMK, V-2, and L were maintained on glass in Eagle's medium(10) containing 10% horse serum. Cultures of Chang's conjunctiva, H. Ep. #2 and V-1 were maintained in Eagle's medium supplemented with 15% horse serum and 5% human serum. The experimental medium for all cell lines was Eagle's medium supplemented with 10% horse serum. Experimental conditions were similar to those previously reported(2). Four- to 6-day stock cultures were trypsinized and subcultured in 60 mm Petri plates. After 24 hours appropriate concentrations of inhibitor and/or reverser, sterilized by Millipore filtration, were added and the plates incubated in presence of CO_2 for 7 days. Medium and appropriate compounds were renewed after 4 days. Cellular growth was measured as total protein (after triple washing) by the colorimetric method of Oyama and Eagle(11). To determine degree of inhibition and subsequent reversal, growth in control plates (no additions) at 7 days was taken as 100% with protein values at zero time representing zero growth. Control plates exhibited a 7- to 12-fold increase in protein during 7-day incubation. Unless otherwise noted, experiments were carried out twice, in triplicate. In measurement of growth rate in cell colonies 5×10^3 cells were plated on 60 mm Petri plates engraved on outer surface with a numbered grid. After incubation for 24 hours the position of approximately 30 cells (with respect to engraved grid) was noted and appropriate compounds were added. At 24 hour intervals, number of cells/colony for each of the 30 colonies was determined microscopically at 39 or 156 × using inverted microscope. *Chemical Procedures.* In experiments to measure incorporation of exogenous thymine, cells were grown on surface of large Blake bottles. Thymine-2-C^{14} (1.2×10^6 cpm/μmole) and appropriate inhibitors (5-fluorouracil and 5-fluorodeoxyuridine) were added when growth was heavy (3 days prior to confluent growth) and incubation was continued 3 days. The

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‡ Present address: Biochem. Sec., Armed Forces Inst. of Pathology, Bethesda, Md.

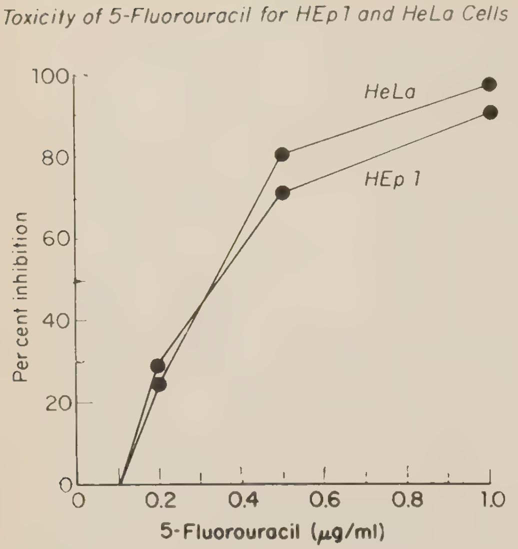

FIG. 1. Toxicity of 5-fluorouracil for H.Ep. #1 and HeLa cells. Growth measured as cell protein after incubation for 7 days.

cells were washed 3 times with saline and removed from the glass by trypsinization. The cells were rewashed (3 times) in saline and extracted with two 5 ml volumes of cold 2% perchloric acid. The residue was extracted twice with absolute ethanol and twice with ether. Hydrolysis was carried out in 0.15 ml of hot 70% perchloric acid (boiling water bath) for 45 minutes. The free thymine was separated by descending paper chromatography (Whatman #1) using isopropanol-HCl (12) followed by ascending chromatography using water-saturated butanol(12). After elution from the paper, the specific activity of thymine was determined. Radiocarbon activity was based on flow-gas counter measurements with aluminum planchets. Concentration was calculated from optical densities at absorption maxima (pH 2). Elution from control paper strip was used as a blank. Purity of thymine was checked by optical density ratios at 250/260 and 280/260 mμ.

Results. The results shown in Fig. 1 indicate that 5-fluorouracil is equally effective in inhibiting growth of both H. Ep. #1 and HeLa cells. However, attempts to reverse growth inhibition by 5-fluorouracil gave different results for the 2 cell strains (Table I). Thymine or thymidine did not reverse growth inhibition of H. Ep. #1 cells but partially reversed growth inhibition of HeLa cells. The latter is in agreement with a report by Ackermann(4). A 10-fold increase in concentration of thymine or thymidine did not appreciably increase reversal of growth inhibition of HeLa cells.

To determine whether this 20-30% overall growth in presence of 5-fluorouracil and thymine indicated a normal growth rate by a fraction of the cell population or a reduced growth rate for the whole population, clonal growth rate studies were carried out. Results of a typical experiment are shown in Fig. 2 where each point represents a single colony. Despite the scatter it can be seen that growth rate of clones in the presence of 5-fluorouracil and thymine is less than that for untreated cells. Since each cell selected at start of experiment was accounted for, the viability of cells grown in presence of 5-fluorouracil and thymine was not significantly lower than that for cells grown in the control medium. From the curve for cells grown in 5-fluorouracil alone, it can be seen that concentration of 5-fluorouracil in this medium was sufficient to prevent growth in absence of a reverser.

In H. Ep. #1 cells, isotopic tracer studies had demonstrated that exogenous thymidine was utilized to a greater extent for DNA thymine when the pathway leading to *de novo* formation of the thymine moiety was blocked by addition of 5-fluorodeoxyuridine to the culture medium(2). It was therefore of interest to compare the extent to which exogenous thymine (supplied in the medium) is utilized in formation of DNA thymine under conditions of normal cell growth. In the experiment summarized in Table II (5-fluorouracil absent) cell growth was approximately equal for each cell line during the 3 day incubation with

TABLE I. Effect of Thymine and Thymidine on Growth Inhibition of H.Ep. #1 and HeLa Cells by 5-fluorouracil (1 μg/ml).

	Conc. (μg/ml)	% reversal of growth inhibition (± avg dev.)	
		H.Ep. #1	HeLa
Thymine	1	0	4 ± 4
	10	0	21 ± 4
	100	0	26 ± 6
Thymidine	10	0	20 ± 5
	100	0	30 ± 12

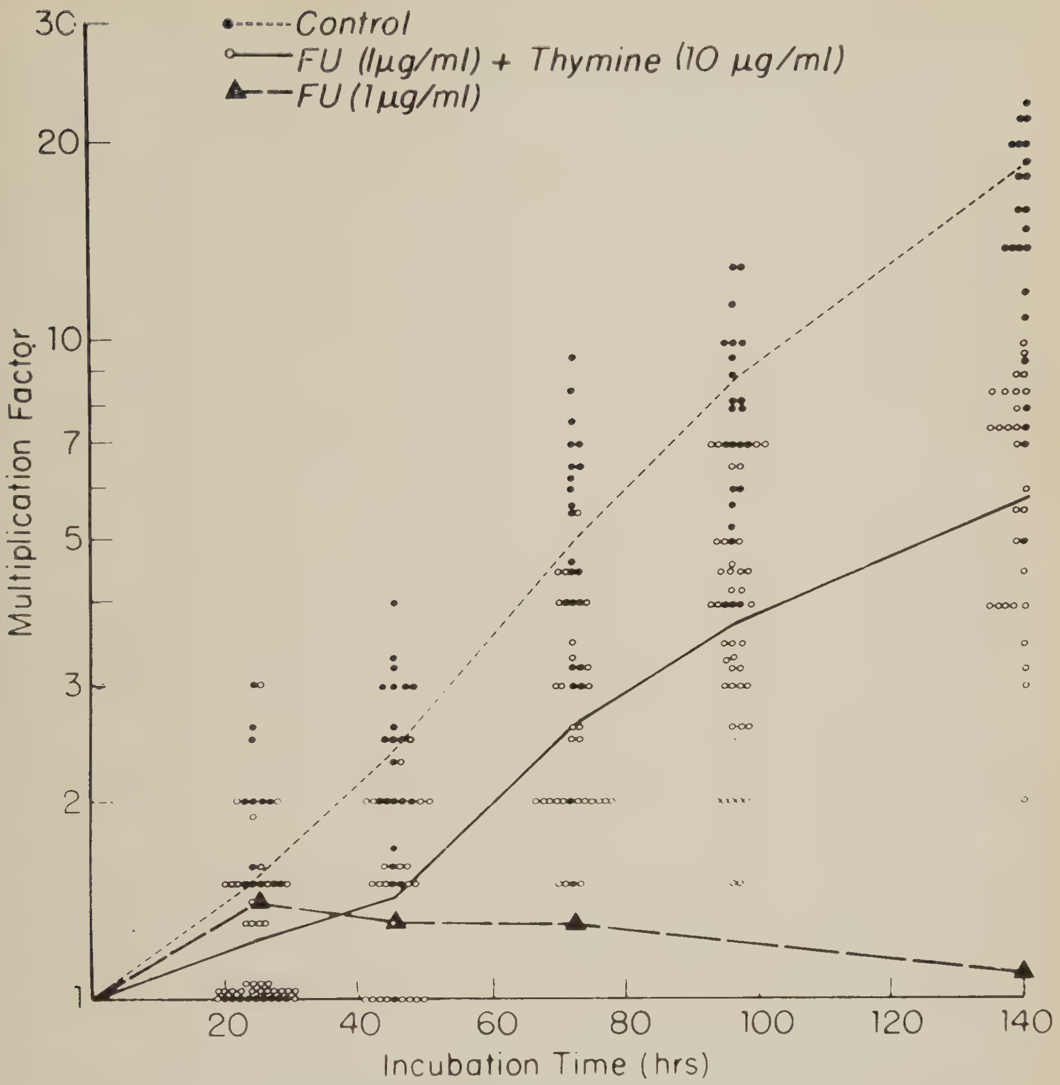

FIG. 2. Effect of 5-fluorouracil and thymine on clonal growth rate of HeLa cells. Each point represents No. of cells in a single colony. In the case of FU, only mean values are plotted. Lines are drawn through mean values for each time interval.

thymine-C^{14} present in the medium. Specific activity of DNA thymine in HeLa cells was 3 to 6 times greater than that in H. Ep. #1 cells.

TABLE II. Incorporation of Thymine-2-C^{14}* into DNA of Cells in Culture—Effect of 5-Fluorouracil.

5-Fluorouracil (μg/ml)	Specific activity of DNA thymine (cpm/μmole × 10^{-2})			
	HeLa		H.Ep. #1	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
0	231	517	74	85
1.0	2910	4170	152	168

* Concentration of thymine-2-C^{14}: 50 μg/ml. Specific activity: 1.20 × 10^6 cpm/μmole.

When 5-fluorouracil is present in the medium at 1.0 μg/ml, specific activity of DNA thymine for HeLa cells was increased by factors of 12 and 8 in Exp. 1 and 2 respectively. In these experiments, the exogenous thymine was serving as a partial reverser of growth inhibition as well as a labeled precursor of DNA thymine. In H. Ep. #1 cells specific activity of DNA thymine was increased by a 2-fold factor upon addition of 5-fluorouracil. The

TABLE III. Effect of Several Compounds on Growth Inhibition of H.Ep. #1 and HeLa Cells by 5-Fluorouracil (FU)* and 5-Fluorodeoxyuridine (FUDR).*

Compound	Ratio of conc.: Compound/inhibitor	Reversal of growth inhibition: H.Ep. #1 FU	H.Ep. #1 FUDR	HeLa FU	HeLa FUDR
Thymine	10	—†	—	+	+
	100	—	—	+	+
Thymidine	10	—	+	+	+
	100	—	+	+	+
Uracil	10	—			—
	100	—	—	+	—
Uridine	10		—		—
	100	—	—	—	—
Deoxyuridine	10	—	—	—	—
	100	—	+	+	+
Cytosine	10	—	—	—	—
	100	—	—	—	—
Cytidine	10	—		—	—
	100	—	—	±	—
Deoxycytidine	10	—	—	±	±
	100	—	—	+	+
5-Methyldeoxycytidine	10	—	+	+	+
	100	—	+	+	+

* Concentration FU: 1 μg/ml; FUDR: 0.1 μg/ml. These concentrations of FU and FUDR completely inhibit growth of both cell lines.

† Reversal of inhibition: (—) 0-9%, (±) 10-19%, (+) 20-100%.

extent of utilization of exogenous thymine for HeLa cells in presence of 5-fluorouracil is significantly high. Specific activity of DNA thymine (4.2 x 10^5 cpm/μmole) was 35% of that for exogenous thymine.

Several compounds related to metabolites involved in nucleic acid synthesis were tested for ability to reverse inhibition by 5-fluorouracil and 5-fluorodeoxyuridine in H. Ep. #1 and HeLa cells (Table III). As previously reported(2) inhibition by 5-fluorouracil of H. Ep. #1 cells could not be reversed by any of the metabolites tested. Inhibition by 5-fluorodeoxyuridine could be completely reversed by thymidine or 5-methyldeoxycytidine at 10 times the concentration of inhibitor (10×) while deoxyuridine was effective at 100×. In HeLa cells, inhibition of 5-fluorouracil could be partially reversed at 10× by thymine, thymidine and 5-methyldeoxycytidine, and to a lesser extent by deoxycytidine. At 100× uracil and deoxyuridine effected a partial reversal. Uracil and cytosine at 100× did not reverse inhibition by 5-fluorodeoxyuridine, but did effect partial reversal of inhibition by 5-fluorouracil.

In view of differences between H. Ep. #1 and HeLa cells it appeared of interest to compare the response of other mammalian cell lines. The results with 6 cell lines are reported in Table IV. In all cases complete growth inhibition resulted at concentrations of 5-fluorouracil and 5-fluorodeoxyuridine of 1.0 and 0.10 μg/ml respectively.

All strains tested, except Chang's conjunctiva and V-2, exhibited a response similar to H. Ep. #1 cells in that inhibition by 5-fluorouracil could not be reversed by either thymine or thymidine. Thymine or thymidine effected an 18 to 24% reversal of growth inhibition with Chang's conjunctival cells. Addition of thymidine resulted in 35% reversal in V-2 cells. Partial reversal by thymine of inhibition by 5-fluorodeoxyuridine was observed only in H. Ep. #2 cells. Thymidine effectively reversed growth inhibition by 5-fluorodeoxyuridine in all cell lines tested.

Discussion. Studies in several laboratories strongly support the conclusion that the principal site of growth inhibition by 5-fluorode-

TABLE IV. Effect of Thymine and Thymidine on Growth Inhibition of Several Mammalian Cell Lines by 5-Fluorouracil and 5-Fluoro-2′-deoxyuridine.

Cell line	Compound tested for reversal	% reversal of growth inhibition: 5-Fluorouracil*	5-Fluoro-2′-deoxyuridine*
Chang	Thymine*	±†	—†
	Thymidine*	±	+
H.Ep. #2	Thymine	—	±
	Thymidine	—	+
V-1	Thymine	—	—
	Thymidine	—	+
V-2	Thymine	—	—
	Thymidine	±	+
AMK	Thymine	—	—
	Thymidine	—	+
L	Thymine	—	—
	Thymidine	—	+

* Concentrations: 5-fluorouracil, 1.0 μg/ml; 5-fluorodeoxyuridine, 0.1 μg/ml; thymine, 100 μg/ml; thymidine, 10 μg/ml.

† Scoring of reversal of growth inhibition: —, no reversal (less than 10% reversal). ±, partial reversal (less than 35% reversal). +, complete reversal (greater than 70% reversal).

oxyuridine involves interference with steps leading to *de novo* synthesis of the thymine moiety, probably at the level of thymidylate synthetase(2,13,14,15,16,17,18). Complete reversal by thymidine of growth inhibition by 5-fluorodeoxyuridine in all cell lines studied, as contrasted with partial reversal by thymine observed in one of the cell lines studied, is in accord with the demonstrated greater utilization of thymidine rather than thymine for DNA thymine synthesis in mammalian cells (19,20,21). Tracer studies in Table II suggest that thymine is utilized to a greater extent for DNA thymine synthesis by HeLa as compared with H. Ep. #1 cells. In the presence of 5-fluorouracil, thymine-C^{14} was extensively utilized for DNA thymine synthesis by HeLa cells (in one experiment specific activity of DNA thymine was 35% of that of thymine added to culture medium). Further studies may indicate the contribution of differences in catabolism of thymine and its derivatives in addition to differences in the capacities of these cells to synthesize thymidine and thymidylate from exogenously supplied thymine (22).

Although 5-fluorouracil inhibits growth of HeLa and H. Ep. #1 cells approximately equally over a range of inhibitor concentrations (Fig. 1) differences between these 2 cell lines in reversal of growth inhibition by thymine and thymidine are clearly demonstrated by results described above. While the major site of growth inhibition by 5-fluorodeoxyuridine in these experiments appears to be well established, as stated above, inhibition by 5-fluorouracil of H. Ep. #1 cells probably includes conversion to acid soluble ribonucleotides, incorporation into ribonucleic acid and interference with its synthesis, in addition to interference with *de novo* synthesis of the thymine moiety(15,16,17). The partial reversal by both thymine and thymidine of inhibition by 5-fluorouracil in HeLa cells (and not in H. Ep. #1 cells) suggests that the mechanism of inhibition involves to a lesser extent in HeLa cells those metabolic pathways not directly involving synthesis of the thymine moiety. Differences among various cell lines in catabolism of fluorouracil and its derivatives as well as anabolic steps leading to ribonucleosides and phosphorylated derivatives may underly the variations in reversal of growth inhibition among different cell lines(22).

Differences between H. Ep. #1 and HeLa cells with respect to reversal of inhibition are also reflected in attempts to reverse inhibition with other nucleic acid metabolites (Table II). Reversal of inhibition by 5-fluorodeoxyuridine in HeLa and H. Ep. #1 and 5-fluorouracil in HeLa by 5-methyldeoxycytidine suggest that this compound might be utilized in pathways leading to synthesis of DNA thymine in mammalian cells(2). A similar role in microbial systems has been demonstrated by Cohen and Barner(23).

Summary. Growth inhibition of HeLa and H. Ep. #1 cells by 5-fluorouracil and 5-fluorodeoxyuridine and reversal of inhibition by thymine and thymidine have been studied. Growth inhibition curves with 5-fluorouracil are similar in both cell lines while reversal studies reveal several differences. Thymine partially reverses inhibition by 5-fluorouracil and 5-fluorodeoxyuridine in HeLa but not in H. Ep. #1 cells. Thymidine reverses inhibition by 5-fluorodeoxyuridine in both H. Ep. #1 and HeLa cells but reverses inhibition by 5-fluorouracil only in HeLa cells. Partial reversal (about 30%) of inhibition by 5-fluorouracil in HeLa cells represents more closely a reduced growth rate by the whole cell population rather than a normal growth rate by a fraction of the population. Utilization of thymine-2-C^{14} for DNA thymine synthesis in HeLa cells was significantly increased in presence of 5-fluorouracil.

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Early Permeability Effects of Estradiol on Castrate Rat Uterus.* (25673)

I. D. K. HALKERSTON, J. EICHHORN, M. FEINSTEIN, E. SCULLY AND O. HECHTER
Worcester Foundation for Experimental Biology, Shrewsbury, Mass.

The view that the primary mechanism of action of various hormones at a cellular level may involve regulation of permeability processes in target cells is being investigated in this laboratory. We previously studied cellular permeability effects of ACTH upon the adrenal(1) and have reported certain similarities between this action of ACTH and that of insulin on muscle fibers(2,3). In the present work we examined the early effects of estradiol-17β upon permeability processes in castrate uterus (using C^{14} labeled "non-utilizable" sugars, α-aminoisobutyrate (AIB), and Na^{22}) to determine whether the primary action of estrogen might be related to increasing substrate entry into the cell. Alterations in carbohydrate metabolism in isolated uteri following estrogen administration *in vivo,* have been ascribed to increased transfer of glucose into the cell(4). The effect of estrogen to increase amino acid incorporation into uterine protein under similar conditions(5) might likewise be considered as the result of increased amino acid transport, inasmuch as Noall *et al.*(6) have shown that estrogens administered 20 hours previously specifically increase accumulation of AIB in the uterus. Our results indicate that estradiol-17β does not increase uterine permeability to substrates at a time when changes in uterine metabolism of carbohydrate and protein are evident.

Methods. Sprague-Dawley rats (200-250 g), ovariectomized 4-7 weeks previously, were functionally nephrectomized at time of experiment to maintain relatively constant blood levels of radiotracers(1). In most cases they were adrenalectomized 48 hours prior to experimentation to remove possible inhibition by corticoids (liberated during stress of kidney ligation) of uterine response to estrogen(4,7). After ligation of both kidney pedicles under

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Avertin anesthesia, one of the following tracer substances was injected subcutaneously: inulin-C^{14} (carboxyl label); sucrose-C^{14} (uniformly labeled); D-xylose-1-C^{14}; α-aminoisobutyric acid-1-C^{14} or Na^{22}. Ninety minutes after tracer injection, plasma from aortic blood was obtained and at the same time uterus and certain other tissues were excised, weighed and prepared for radioactive assay as described elsewhere(1). *Uteri were slit longitudinally and blotted between filter paper in standard manner before weighing to remove luminal fluid.* Effects of estradiol-17β were determined 1.5 and 6 hours after single injection (10 μg in 1 ml saline-PO_4 into tail vein). For the 1.5 hour interval the hormone was given immediately prior to radioactive tracer. In those cases where estradiol was given for 6 hours, the hormone was administered 4.5 hours before kidney ligation and tracer injection.

Results obtained in functionally nephrectomized, adrenalectomized, castrate rats are shown in the Table; essentially similar results were obtained when adrenals and/or kidneys were not removed. Estradiol produced the expected increase in wet weight and water content of the uterus after 6 hours, but no significant changes were detected at 1.5 hours. The Table also shows percent volume of distribution (counts/g wet tissue/counts/ml plasma) of various tracer substances studied in uterine tissue, with the italicized values in the Table representing volume of distribution of radiotracers in total tissue water. No change was seen 1.5 hours after estradiol administration in distribution of sucrose, Na^{22}, D-xylose or AIB; either in terms of wet weight or total tissue water. The inulin space was significantly increased 90 min after estrogen, but was not further increased at 6 hours. Six hours after estrogen, there was a small but significant increase in distribution of D-xylose and AIB over the control level, which was not accompanied by significant change in sucrose or Na^{22} spaces.

Discussion. To assess the significance of the results in the Table in terms of entry into cell water, it is necessary to have a measure of extracellular space. Inulin, sucrose (and Na to a lesser extent) are excluded from the

TABLE I. Effect of Estradiol-17β on Weight and Water Content of Castrate Rat Uterus and Volume of Distribution of Radiotracers.*

Interval after estradiol inj., hr	Wet wt, mg	Water content, ml/g	Vol of distribution (%/g) in tissue ± S.E. and No. of rats (Italicized: Vol of distribution in total tissue water)				
			Inulin-C^{14}	Sucrose-C^{14}	Na^{22}	D-xylose-C^{14}	AIB
0	55 ± 1.5 (67)	.76 ± .003 (5)	43.8 ± 1.4 (19) *58*	57.6 ± 1.4 (30) *76*	58.4 ± 2.1 (7) *76*	69.7 ± 1.9 (32) *92*	102.8 ± 7.1 (13) *135*
1.5	56 ± 2.9 (20)	.76 ± .007 (5)	54.7† ± 1.4 (9) *72*	54.8 ± 1.5 (16) *72*	61.1 ± 3.4 (5) *80*	71.2 ± 3.6 (16) *94*	107.6 ± 13.2 (7) *142*
6.0	87 ± 4.8 (29)	.83 ± .015 (6)	55.9† ± 2.3 (6) *67*	61.1 ± 2.2 (6) *74*	56.5 ± 3.8 (4) *68*	78.9‡ ± 2.5 (9) *95*	147.8‡ ± 10.0 (8) *178*

* Dose levels: Inulin-C^{14} (New England Nuclear) 1.3 μc (0.95 mg); sucrose-C^{14} (New England Nuclear) 1.29 μc (0.17 mg); Na^{22} (Nuclear Science and Engineering Corp.) 1.0 μc (trace); D-xylose-1-C^{14} (Nat. Bureau of Standards) 1.09 μc (0.48 mg); AIB-1-C^{14} (Volk Radiochemicals) 0.71 μc (0.94 mg). Dose levels per 100 g body wt.

† Level of significance: $p < .001$.

‡ Level of significance: $p < .01, > .001$.

cell water of many tissues and have accordingly been widely employed as measures of the extracellular compartment of tissues. In uterus, however, all of these substances distribute in such a large fraction of the total tissue water that it is not possible to assume that they are, in fact, excluded from the cell water. In untreated castrate uterus, inulin distributes in 58% of total tissue water and likewise sucrose (and Na^{22}) distribute in a very large fraction of total uterine water (76%). Comparable values for sucrose distribution in the total water of other tissues from these same rats are 25, 19 and 15% for intestine, diaphragmatic muscle and thymus respectively. All of these tissues show the same order of wet wt. : dry wt. ratios, and furthermore the histological appearance of castrate uterus makes it difficult to visualize an extracellular space of the order suggested by inulin or sucrose. Accordingly, it would appear that in the castrate rat uterus, inulin, sucrose and Na^{22} enter into cells; the space occupied by inulin, in absence of estrogen, being somewhat less than that of sucrose or Na^{22}. Cole(8) using thiocyanate as a measure of extracellular volume in uterus, found a tissue distribution of 29%, a value lower than that indicated by inulin which would be consistent with the idea that inulin penetrates into many of the cells in castrate uterus. Pappius and Elliott(9) experienced similar difficulties in attempting to define the extracellular volume in cerebral cortex and likewise were forced to assume penetration of sucrose and Na into the cell.

Independent of whatever value is taken for extracellular volume, our results demonstrate that in absence of estrogen, D-xylose is not excluded from uterine cell water, and AIB is accumulated against an apparent concentration gradient. Where hormones do increase permeability of target cells to sugars, as in the case of insulin and ACTH, it is to be noted that barriers exist which limit the entry of D-xylose, such that the sugar is excluded from intracellular regions which are subsequently made available when hormone is present. Accordingly, the fact that estrogen has no effect on D-xylose entry in uterus is not surprising since barriers which exclude sugars do not appear to be present in this tissue. In similar fashion, estrogen has no effect on AIB transport equivalent to the early stimulation of uptake by ACTH in adrenals and insulin in muscle. The enhanced uptake of AIB which can be detected at 6 hours and is well established at 20 hours(6), appears to be one of many secondary responses of the uterus to estrogen administration. Six hours after estradiol administration, many secondary events have already taken place(5).

If the effects of estrogen on excised uterine metabolism(4,5), clearly shown one hour after intravenous injection of the hormone, were due primarily to an action of estrogen to increase substrate entry, then one would expect to see changes in sugar and amino acid permeability 1.5 hours after hormone administration. The fact that such changes do not occur, coupled with the lack of exclusion of substrates from uterine cells, indicates that estrogen *via* some primary mechanism activates enzyme systems concerned in carbohydrate and protein metabolism independently of increased substrate entry into the cell.

Summary. The influence of estradiol-17β on distribution of certain non-metabolizable sugars, the "non-utilizable" amino acid (AIB) and Na^{22} in uterus of functionally nephrectomized castrate rats was examined. Uterine cells did not exclude D-xylose-1-C^{14} in absence of estradiol, and no effect on uterine permeability to D-xylose-1-C^{14} or α-aminoisobutyric acid-1-C^{14} was observed 1.5 hr after hormone administration, at which time changes in uterine metabolism of carbohydrate and protein have been established.

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Selenium and Vit. E as Related to Growth and White Muscle Disease in Lambs.* (25674)

J. E. OLDFIELD, O. H. MUTH AND J. R. SCHUBERT (Introduced by J. S. Butts)
Depts. of Dairy and Animal Husbandry, Vet. Medicine and Agric. Chemistry, Oregon State College, Corvallis

Interest has been focused recently upon the effects of 2 dissimilar entities—selenium and Vit. E—in overcoming certain muscular dystrophies. Those dystrophies caused by feeding experimentally-compounded, Vit. E-deficient diets have been cured by addition of dietary Vit. E in rabbits(1), guinea pigs(2), mice(3), lambs(4) and chicks(5). Vit. E has also proven effective when added to dystrophogenic diets high in unsaturated fats(6). On the other hand, minute amounts of selenium have been found to protect chicks on torula yeast diets against exudative diathesis (7) and similarly-fed rats(8) and mice(3) against hepatic and multiple necroses, respectively. In Oregon, 0.1 part/million of selenium in dry matter of ewes' diet has protected lambs from white muscle disease (WMD) when their mothers were fed pre-partum on a "causative" ration(9). Some syndromes provide evidence that Vit. E and selenium may act in distinctly different ways. Thus, studies of Schwarz and associates showed that Factor 3-active selenium compounds were ineffective against rabbit muscular dystrophy on Vit. E-free diets(10) and inactive in rat resorption-gestation technic for Vit. E bioassay(11). Further, the Oregon workers showed that neither parenteral nor oral administration of Vit. E to ewes pre-partum would prevent WMD in their lambs, while oral selenium proved highly protective(9). Since previous studies suggest a growth stimulation in chicks(12,13) and in lambs(14) resulting from sub-toxic levels of selenium supplementation, it has become of interest to investigate the effects of Vit. E and selenium upon growth and dystrophy prevention simultaneously in sheep exposed to diets conducive to WMD. Additionally, it was desired to confirm and extend earlier findings as to control of WMD by administration of selenium. This paper reports on growth rate and incidence of WMD in lambs whose mothers received a dystrophogenic Ladino hay diet with and without selenium and Vit. E.

Materials and methods. Forty-eight mature, pregnant, crossbred ewes were randomly assigned to 4 lots housed in identical pens bedded with wood shavings. Lot 1 was fed the basal ration of 4 lb. Ladino clover hay and ¼ lb. ground oats per head daily. Lot 2 and Lot 3 ewes received the same basal ration; in addition the Lot 2 lambs received 1.40 mg Se, as aqueous Na_2SeO_3 inj. intramuscularly, while Lot 3 lambs were each given 2,000 IU Vit. E orally. Lot 4 ewes received the basal diet supplemented with 0.1 ppm selenium as Na_2SeO_3 calculated on basis of total ration dry matter and added to the ground oats. Diet treatments commenced about third month of gestation and continued until lambs were 6 weeks old. All groups received water and iodized salt, *ad lib*. Lambs in Lot 2 received selenium in 2 injections: the first, at 1 day of age, supplied 0.28 mg, while the second, at 14 days, supplied 1.12 mg Se. The sum of these doses was calculated to provide 0.2 ppm Se in estimated dry matter intake for the first 6 weeks of life. Vit. E was given in aqueous suspension, (Type "F-50" containing 50,000 IU Vit. E/lb, supplied by Distillation Products Industries, Rochester, N.Y.) in 2 equal doses at 1 and 4 days of age. The lambs were weighed at birth, 2 weeks and 6 weeks of

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TABLE I. Effects of Selenium and Vit. E on Incidence of WMD and Growth in Lambs.

Lot	Total lambs	Lambs with gross WMD lesions	Avg wt (lb) of lambs at Birth	2 wk	6 wk	Gain (lb), birth-6 wk
1	20	16	9.99	16.62	25.31	15.32
2	17	0	9.30	17.53	30.14	20.84*
3	18	0	9.07	16.40	26.40	17.33
4	10†	0	10.35	20.53	37.50	27.15*

* Significantly greater than Lot 1 ($P < .05$).

† In addition to those listed, 2 sets of triplet lambs were born in Lot 4 and lost due to difficulties at parturition. No lesions of WMD were apparent.

age. All surviving lambs were slaughtered at 6 weeks and examined for lesions.

Results. All treatments (Lots 2, 3, and 4) were effective in protection against clinical symptoms and gross lesions of WMD (Table I).

In this experiment oral Vit. E, given directly to lambs, was protective, whereas our earlier experiences showed that Vit. E given ewes pre-natally was not(9), suggesting possible difficulties in placental transfer of the vitamin. Growth of lambs receiving Vit. E, however, was not significantly different from that of untreated controls (Lot 1). On the other hand, growth of both lots of lambs receiving selenium was significantly greater than that of controls ($P<0.05$) and growth of those lambs whose mothers received oral selenium was significantly greater than that of lambs which received selenium by injection ($P<0.05$). This difference is interpreted as indicating that the ravages of WMD begin pre-natally and may be prevented by pre-natal administration of selenium to the ewe. Selenium given post-natally in amounts listed, however, while arresting the course of WMD, does not completely overcome growth interference.

Growth responses resulting from selenium administration in this study appear to be further confirmation of the role of selenium as an essential nutrient, *per se,* necessary for normal growth even in absence of WMD lesions.

Summary. 1) Selenium, given pre-natally *per os* to ewes or post-natally by injection to lambs, prevented WMD and resulted in increased growth of lambs as compared to untreated controls. 2) Growth response to selenium fed pre-natally was greater than that to selenium injected post-natally. 3) Massive doses of Vit. E at birth protected lambs from WMD, but did not result in improved growth. 4) Evidence suggests an essential role for selenium in supporting growth of lambs.

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Vitamin Levels in Normal Cerebrospinal Fluid.* (25675)

HARRY SOBOTKA, H. BAKER AND O. FRANK
Dept. of Chemistry, Mount Sinai Hospital, N. Y. City

While studying the role of vitamins in various metabolic disorders, we became interested in the vitamin content of cerebrospinal fluid (CSF). We found in the literature only a few scattered data on ascorbic acid in CSF(1). We therefore investigated the content of those water-soluble components of the B-complex for which we had developed microbiological assay methods: folic acid(2), folinic acid (citrovorum factor)(3), pantothenic acid(4), thiamine(5), cyanocobalamin(6), and the Vit. B_6-group (to be published).

Methods. The microbiological assay methods have been described. They require only small quantities of CSF, such as were available from patients undergoing a spinal tap prior to spinal anesthesia for minor surgical conditions.

Results. The ranges for a group of 43 subjects are given in Table I. For comparison the ranges for whole blood are also listed. These data permit evaluation of the quotient of titre in blood over titre in CSF.

Discussion. Pantothenic acid appears to be evenly distributed between the 2 liquids as if it had no barrier to contend with. Folic acid likewise gives a ratio near unity for its concentrations in CSF and blood. Thiamine is 3-5 times less concentrated in CSF than in blood. This quotient assumes a much lower value for cyanocobalamin, which rarely reaches in normal CSF a concentration higher than 30 μμg (= 0.03 mμg), while its titre in blood is 10-30 times higher(5) in CSF than in serum. Its quotient of dilution is of the same range of magnitude as that of the serum proteins of which we find in healthy CSF about 1/250 of their concentration in serum. This would suggest that Vit. B_6 crosses the blood/CSF barrier only attached to serum protein.

These wide discrepancies in the blood/CSF quotients of vitamins are in line with analogous variations in the major blood constituents. CSF is evidently not an ultrafiltrate or transudate of the blood serum. It must be the result of a highly selective secretion by the lining of some regions of its anatomical boundaries. The diffusion at this border must be a 2-way process including absorption as well as secretion, and there must exist 3 barriers: a blood/CSF, a CSF/brain and a blood/brain barrier. A contemplation of the protein pattern of the CSF leads to the conclusion that this protein, about 1/250 of the concentration in blood serum, originates in the general circulation. Since CSF is never without it, but normally contains amounts within the range of 20-40 mg/100 ml, the protein in CSF cannot be due to accidental leakage; nor is its origin by diffusion more likely. One would rather assume the existence of a "window" in the blood/CSF barrier through which limited amounts will constantly pass, possibly in the *regio postrema*. The amounts penetrating into the CSF are small compared to the metabolic turnover. Thus, their concentration in CSF never reaches a static equilibrium. The occurrence and quantity of a given vitamin in CSF is subject to highly individual laws, which are determined by the function and the fate of the vitamin in the CSF.

Summary. Using our microbiological assay methods, we established normal ranges in CSF for folic acid, folinic acid, thiamine, cyanocobalamin, pantothenic acid and the Vit.

TABLE I. Vitamin Content of Normal CSF and Whole Blood.

Vitamin	M.W.	CSF	Blood	Quotient, blood/CSF
		mμg/ml		
Pantothenic acid	219	100-1000	100-1000	1
Folic acid	441	10-30	7-40	1
Folinic acid	473	1-5	0	
Thiamine	337	10-20	25-80	3-5
Cyanocobalamin	1387	0-0.03	0.3-1.0	30
B_6 group	206	0-0.75	100-180	250

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* Supported by grants from National Multiple Sclerosis Soc. Williams-Waterman Fund, N. Y. City, and from Nat. Assn. of Mental Health.

B$_6$-group. The ratios of CSF levels to blood levels of these vitamins are shown in Table I.

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Macroglobulinemia in a Transplantable Mouse Leukemia.[*] (25676)

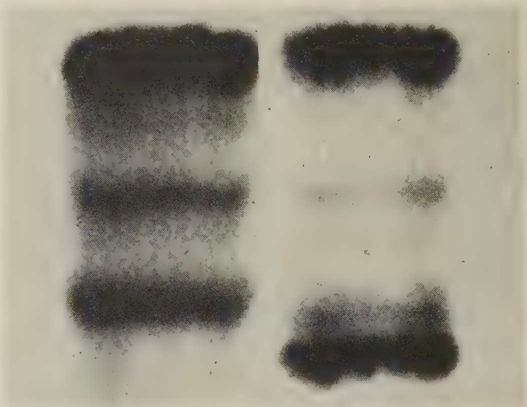
J. CLAUSEN, R. RASK-NIELSEN, H. E. CHRISTENSEN, R. LONTIE AND J. HEREMANS
(Introduced by G. Asboe-Hansen)
Inst. of Biochemistry, Inst. of Pathological Anatomy, Univ. of Copenhagen, Denmark; Lab. of Biochemistry and Clinique Universitaire St. Pierre, Univ. de Louvain, Belgium

Gamma globulin-like paraproteins have been described in various mouse transplantation lines characterized by myelomatous(1,2) or plasmacellular leukemic(3-7) lesions. The subject of this report is the new observation that the paraprotein of a new transplantation line of leukemia appeared to be a macroglobulin and that the microscopic lesions seen in this line are comparable to those described in Waldenström's macroglobulinemia in man.

Material and methods. Methods of total protein estimation, paper- and immunoelectrophoresis, have been described(7). Ultracentrifugation was performed in a Spinco ultracentrifuge (Model E equipped with a rotor temperature indicator and control unit at 59,780 rpm at 20°C) on serum samples, diluted with 2 volumes of 0.9% NaCl. *The transplantation line,* originated in a 28-months old, untreated (CBA x DBA/2) F$_1$ mouse(8) with enlargement of lymph nodes and splenomegaly. Following subcutaneous inoculation of leukemic tissue, percentage of takes through 12 transfer generations was 100, survival time being 6 to 10 weeks, gradually shortening to 4 to 6 weeks.

Results. Total serum protein was found reduced to 3-4.5 g% (normal values 6.1-6.7 g %).

Serum protein pattern in (a) *paper electrophoresis* (Fig. 1) showed a pathologic globulin fraction of fast gamma mobility, in the first 5 passages constituting up to 40% of total protein; gradually fading, it disappeared in the tenth passage. b) *Immunoelectrophoresis* (Fig. 2) revealed an abnormal precipitation bow in the beta-1-beta-2- area located



6.1 g %	Total protein	5.1 g %
60%	Alb.	29%
5%	α_1	6%
9%	α_2	10%
15%	β	15%
	Path. fr.	35%
11%	γ	5%

FIG. 1. Paper electrophoresis of normal mouse serum (left), and of serum from a mouse No. 2420 of fourth transfer (right) characterized by large, abnormal gamma fraction.

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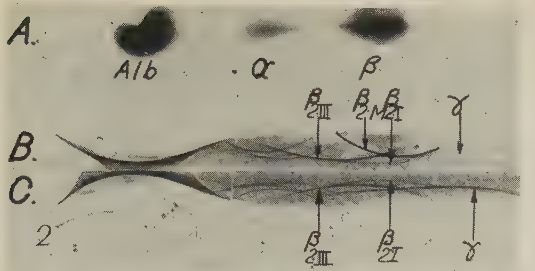
[*] This investigation supported by grants from Leukemia Society, N. Y., King Christian X Fd., The Danish Rheumatism Assn., Fund for Promotion of Medical Research, Copenhagen, Fonds National de la Recherche Scientifique, Belgium.

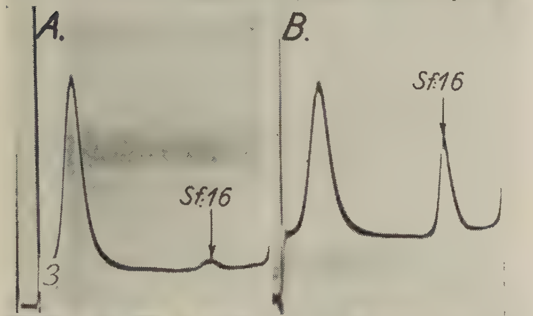
most centrally in all bows, anodically limited by the central filling hole and cathodically superimposed on the beta-2-I (transferrin) bow. In the terminology for human serum this bow is named beta-2-M. In addition, a hypogammaglobulinemia decreasing through the passages to agammaglobulinemia, the latter present from the 5th transfer, and a constantly increased mobility and concentration of beta-2-III bow were observed; this last change, however, is unspecific in that it is seen with all types of malignant growth (6,7, 9,10). In the 7th transfer the pathologic bow was but faintly developed and was invisible from the tenth passage. c) *Ultracentrifugation* (Fig. 3) of serum from a mouse of the fourth transfer showed a large, abnormal globulin peak with sedimentation coefficient 16.2 S constituting 27.5% and located correspondingly to a minimal peak with sedimentation coefficient 16.9 S of normal serum. In conformity with electrophoretic findings, ultracentrifugation of serum from a mouse of the 9th transfer showed an almost normal picture.

Morphologic blood changes. Total number of white blood cells was increased (up to 50,000/cmm) as the result of granulocytosis and presence of numerous (up to 98%) rather small, abnormal cells (Fig. 4a), mostly resembling mature lymphocytoid cells with more or less marked tendency towards plasmacellular differentiation; moreover, a few large dark-staining "blast"-like cells were seen. From the tenth passage lymphocytoid cells appeared more immature, more "blast"-like with dark blue cytoplasm and more reticularly structured nuclei.

Macroscopic lesions in the grafted mice were enlargement of thymus, peripheral and mesenteric lymph nodes, severe hepato- and splenomegaly and perirenal infiltration.

Microscopic tissue lesions were infiltration





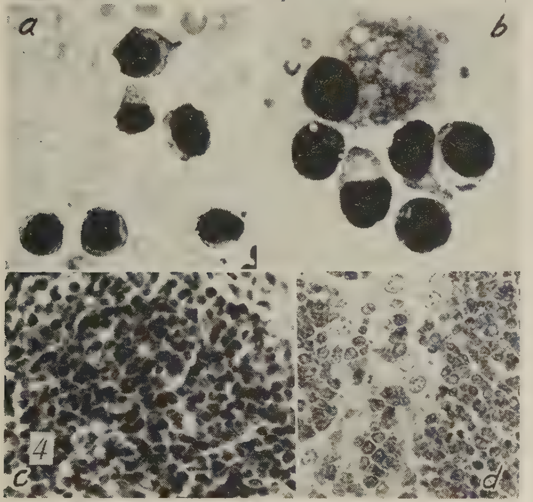


FIG. 2. *A.* Conventional agar electrophoresis of serum from a mouse of third transfer, showing electrically separated fractions. *B.* Immunoelectrophoresis of same serum; presence of a precipitation bow (beta-2-M) not usually seen in normal mouse serum, increased mobility and concentration of beta-2-III and highly reduced extent of gamma bow are evident from comparison with *C.* *C.* Immunoelectrophoresis of serum from normal mouse.

FIG. 3. Ultracentrifugation diagram: *A.* of normal mouse serum, only a small S 16 peak is present. *B.* of serum from a mouse No. 2420 of fourth transfer, showing significant increase of S 16 component.

FIG. 4. *a.* Smear of blood from mouse of ninth transfer — Giemsa stain — × 550; leukemic cells showing large, somewhat eccentric nuclei and scarce, finely vacuolized cytoplasm may be classified as intermediate forms between mouse lymphocytes and plasmocytes. *b.* Imprint preparation of infiltrated lymph node from mouse of third passage, ''naked nuclei'' cells, plasmocytoid lymphocytes and a large reticulum cell with abundant, vacuolized cytoplasm are seen, the latter probably identical with PAS-positive reticulum cells seen in sections. *c.* Tumor at site of inoculation—fourth passage No. 2420—Hem.-Eos. stain—× 225; majority of cells appear as naked nuclei, but diffusely scattered larger reticulum cell with more abundant cytoplasm and paler nuclei are seen. *d.* Liver metastasis from the same mouse as in *c*—methylgreen-pyronine method—× 225 showing chromatin structure and pyroninophilic cytoplasm of the tumor cells.

of typical leukemic localization widely spread, and most markedly developed in the above mentioned organs. Fig. 4 b-d shows morphology of the tumor cells. In imprint preparations of leukemic tissue (Fig. 4 b), the dominating cells were of the same appearance as those seen in the blood. In addition, a varying number of large reticulum-cell like cells were present. Stained with hematoxylin-eosin (Fig. 4 c), the tumor cells presented themselves as naked nuclei. With methylgreen-pyronine staining (Fig. 4 d), they were seen as closely packed, rather small cells with scarce, moderately to heavily pyroninophilic cytoplasm and with relatively large, round or oval nuclei with a distinct nuclear membrane and a fine chromatin network, often with one or 2 small nucleoli. Between the tumor cells large reticulum-cells with vacuolized and/or PAS-positive cytoplasm were located. This picture persisted through 10 transfers. Thereafter the tumor growth showed dedifferentiation into a polymorphous reticulo-sarcomatosis killing the host at a time when only moderate infiltrations had developed; this applied particularly to the liver. In the majority of hosts of all transfers, the Kupffer cells of liver were found PAS-positive, swollen, containing granules, globules and vacuoles.

Discussion. These findings suggest the existence of a macroglobulinemic disease in mice. Localization in paper- and immunoelectrophoresis of the abnormal globulin fraction and its sedimentation coefficient in ultracentrifugation correspond to the findings in macroglobulinemia in man(11-15). The similarity also applies to reduction of the non-influenced immunoglobulins usually seen in paraproteinemic diseases in man(16), and to occurrence of characteristic, more or less plasmacellularly differentiated, lymphocytoid cells (11,17,18), appearing as naked nuclei in hematoxylin-eosin stained sections.

Summary. A transplantable mouse leukemia characterized by presence of a large, macromolecular serum globulin fraction and of more or less plasmacellularly differentiated, lymphocytoid cells is reported. The disease shows definite similarity to human macroglobulinemia.

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Device for Inhalation Exposure of Animals to Spores. (25677)

Willard R. Piggott and Chester W. Emmons
U. S. Dept. of H. E. W., P.H.S., Nat. Inst. of Allergy and Infectious Diseases, N.I.H., Bethesda, Md.

Several systemic mycoses begin as upper respiratory or pulmonary infections following inhalation of fungi. The fungi, presumably, are inhaled as spores in air-borne dust from colonies of pathogenic fungi growing in the patient's environment. In experimental studies it sometimes is desirable to simulate these conditions of natural exposure to fungi. Elaborate and expensive instruments have been designed to disperse estimated numbers of spores or particles in air to which animals are experimentally exposed. Recognized disadvantages of these include cost, large size, inconvenience of operation and contamination of the laboratory. The quantitative aspects of exposure are unpredictable because counts of the spores per unit volume of air in such an instrument do not measure numbers which actually enter the lungs.

Methods. The device described herewith is at first a culture flask and subsequently an exposure chamber. It overcomes the disadvantages pointed out above, and simultaneous exposure of 10 mice permits an adequate sampling of animals for accurate estimation of number of viable spores which actually enter the lungs. Its design can be modified to accommodate larger animals and for use with atomized suspensions of microorganisms or for dusts of selected particle size.

Results. Fig. 1 is a photograph of the device in use as a culture flask. It is a 1 liter heavy wall pyrex flask with 10 tubular side arms fused (ring-sealed) at a 30° angle above the horizontal into ports spaced equidistantly around the flask at a level of 4 cm above the base. Each side arm is a pyrex tube of 2 mm wall thickness, 21 mm inside diameter and 14 cm length, constricted to a 1 cm opening at one end. This end extends about 2 cm into the flask beyond the seal. The mouth of the flask and of each side arm is closed by a cotton stopper. The lower end of each side arm also may be closed by a cotton stopper on an applicator stick which extends beyond the distal end of side arm. The flask is filled to a depth of 2-3 cm with an agar medium, it is sterilized in the autoclave, the agar surface is seeded with a suspension of spores, the flask is incubated until mature spores are abundant and the device is then ready for its role as an exposure chamber.

Fig. 2 is a semidiagrammatic drawing showing (for clarity) only 2 of the 10 side arms. It illustrates use of the device as an exposure chamber by replacement of the cotton stopper in the mouth of the flask by a unit for creating air currents over the surface of the culture. This is a #10 stopper through which pass 2 glass tubes. The upper end of the shorter tube is bent at a right angle and attached to a rubber balloon capable of expansion to 100 ml capacity before elastic changes. The longer tube is bent to a right angle but at a point above the first so that it can be rotated. It is attached by rubber tubing to a 100 ml syringe. The tube is held in position by 2 rubber collars (not shown in the drawing) and the portion which passes through the stopper is lubricated with glycerine. The lower end of this tube is curved to direct a jet of air across the surface of the fungus culture. This unit is wrapped in paper and sterilized in the autoclave.

In preparation for use of the device as an exposure chamber, and before insertion of the air-circulating unit, a mouse is placed in each of the side arms. It is pushed to the bottom so that its nose fills and extends beyond the open end of the tube and it is held in place by a cotton plug. The upper end of the side arm is then closed by a rubber stopper. The air circulating unit is now put in place.

When the apparatus is fully assembled with mice in place an attendant produces air turbulence over the culture by pumping 50 ml of air into and out of the chamber by regular strokes of the syringe plunger. A second attendant, by rotation of the tube through which air is pumped, directs an air jet uniformly over all parts of the culture to disperse spores throughout the chamber. Changes in

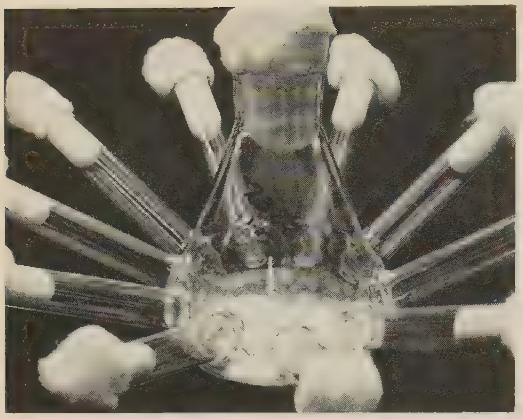

FIG. 1. Device in use as a culture flask.

air pressure are minimized by the ballast action of the balloon which must be nearly empty when the syringe plunger is at the 50 ml mark and must be attached in such a way that it never acts as a flap valve to close the tube. It must expand freely so that it will hold 75-100 ml of air without stretching and will thus prevent changes of air pressure within the flask.

Pumping of air should be continued for a period which will vary with species and strain of fungus, number of spores disseminated and severity of exposure desired. The mice should be left in place for 2-3 minutes after air turbulence has subsided to allow partial settling of air-borne spores. The device may be placed in a safety hood during mouse exposure but in use we have not found the experimental fungus in Petri dishes exposed during the entire procedure. The closed system is an essential safety feature of this device but it is obvious that there must be some contamination of at least the anterior portion of the animal's head. Flow of contaminated air over the rest of the body is reduced by the stopper back of the animal and by equalization of pressure achieved by expansion of the balloon. Contamination can be reduced further by swabbing mice with a disinfectant. At completion of exposure mice are removed, cotton stoppers are replaced in side arms and the apparatus is sterilized in the autoclave. If mice must remain within this closed system longer than 10 minutes, a larger flask should be used in the design in order to prevent depletion of O_2 and subsequent changes in the animals' respiration rates and depth of inhalation.

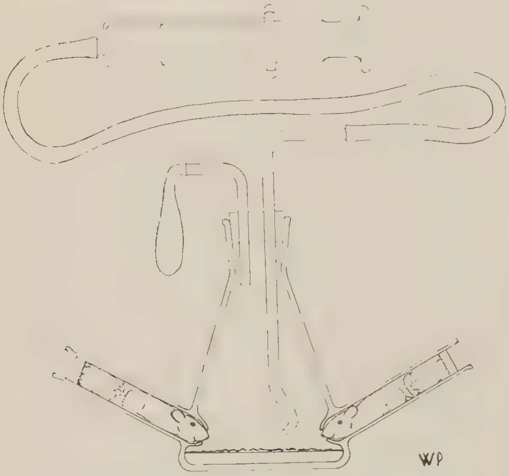

FIG. 2. Use as an exposure chamber.

Our device permits simultaneous exposure of 10 mice to an atmosphere containing dry spores of a fungus. The numbers of viable spores which actually enter the lung can be estimated retrospectively by killing 5 mice taken from alternate side arms, grinding the lungs, plating this tissue and counting fungus colonies after incubation. The instrument can be adapted by changes in design to produce an "atomized" mist of microorganisms in droplets or a dust of selected particle size. Its design can be changed to expose larger animals without loss of its simple, self-contained and safety features.

The device was made to specifications supplied by the authors in the Glass Shop of the Nat. Inst. of Health.

Summary. A culture flask with 10 side arms is converted by insertion of a unit for blowing air across a fungus culture into an exposure chamber. Ten mice or other animals can be exposed simultaneously with minimal contamination to an atmosphere containing dry spores. The design can be adapted to exposure of larger animals and to use with dusts, mists and gases. Superior features of the device are: it is small and portable, it is simple in construction and operation, it is a closed system, and contamination of fur of animals is minimal.

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Plasma Albumin Catabolism in Tissue Slices.* (25678)

Joseph Katz,[†] Alvin L. Sellers, Sybil Golden
Inst. for Medical Research, Cedars of Lebanon Hospital, Los Angeles

Plasma albumin, alpha and beta globulins are known to be synthesized in the liver(1) but the site of breakdown of these plasma proteins is not clearly established. *In vivo* experiments(2) suggest that liver is the main site of albumin degradation, but only limited breakdown of plasma albumin has been demonstrated in isolated perfused rat liver(3,4). Kidney also has been implicated as a site of plasma protein breakdown(5). Roberts and Kelley report extensive catabolism of all plasma proteins by rat liver slices(6). Since this was the only available report of such breakdown, we reinvestigated catabolism of plasma proteins by slices of liver and other organs.

Methods. Animals. Rats of Slonaker-Addis strain weighing about 200 g, and Californian rabbits were used. *Albumin I^{131}.* Plasma albumin was iodinated by procedure of Staub(7). To obtain a preparation of uniform halflife iodoalbumins were "screened" according to McFarlane(8). Homologous iodoalbumin was injected into rats or rabbits, and after 3-4 days animals were bled. Following dialysis against 0.9 NaCl solution to remove non protein I^{131} this screened material was used as radioactive substrate. The iodoalbumin preparations are described in greater detail elsewhere(9). Human iodoalbumin (RISA)® was purchased from Abbott Lab. *Incubation.* Uniform tissue slices of approximately 0.5 mm thickness were prepared with McIlvain-Buddle Slicer(10). Duplicate samples of rinsed and blotted slices were incubated in 20 ml heavy wall graduated glass tubes, that fit a homogenizer pestle. 250 mg of slices were incubated in 2 ml of various media (bicarbonate buffer, phosphate buffer, and serum) at 38° for 3 hours in presence of labelled plasma protein in shaking water bath. The gas phase was either pure oxygen or a mixture of 95% O_2 + 5% CO_2. At end of incubation period, slices were homogenized in the tubes, trichloracetic acid to yield final concentration of 5% was added and the mixture brought to 7 ml volume. Tubes were centrifuged and the clear supernatant used for assay. I^{131} activity was assayed in a scintillation well counter in conjunction with a single channel analyser. In our system one microcurie of I^{131} assayed at approximately 300,000 cts/min, with background count of about 10 cts/min. The extent of plasma protein breakdown was calculated from the difference between activity of trichloracetic acid extract present initially and at end of incubation. Total activity in vessel was obtained from assay of iodoalbumin, and in experiments where iodoalbumin was preinjected (see below), by assaying the trichloracetic acid residue. Proper corrections for counting geometry were applied as needed. *Precision of evaluation of lower limit of breakdown.* The lower limit of non protein I^{131} formation that can be measured depends on a) initial activity of trichloracetic soluble (non protein) fraction and b) background count rate. Unscreened iodoalbumin preparations had about 1% of activity soluble in trichloracetic acid, and screened preparations between 0.1 - 0.4%. In our conditions 10% increase over these initial blanks could be determined. Thus minimal breakdown rates of the order of 0.1% of unscreened preparations and about 0.02% of screened preparations could be estimated with an error of ± 50% or less. Total activity/vessel was usually well above 100,000 counts/minute with unscreened preparations. Thus minimal fractional breakdown rates, discussed above, were well above background count rate. On the other hand in experiments with preinjected albumin (see below) total activities/vessel ranged from several hundred to several thousand counts/minute. In these experiments minimal breakdown rate that can be determined is limited by the value of background count rate (about 10 cts/min) and fractional

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[†] Advanced Research Fellow of Am. Heart Assn.

TABLE I. Breakdown of Screened and Unscreened Homologous Exogenous Iodoalbumin by Liver Slices and Homogenates.

Preparation	Incubation medium	Iodoalbumin breakdown (%) Not screened	Screened
	Rat		
Slices	Serum	2.0	.03
	Bicarbonate	2.8	.08
	Phosphate	2.4	.00
Homogenate	Serum	2.4	.04
	Bicarbonate	3.4	.10
	Phosphate	3.3	.07
	Rabbit		
Slices	Serum	.07	.00
	Bicarbonate	.04	.00
	Phosphate	.02	.00
Homogenate	Serum	.04	.00
	Bicarbonate	.03	.00
	Phosphate	.03	.00

300 mg slices and 0.5 ml whole homogenate (1:1 in 0.25 M sucrose) incubated with 2 ml medium for 3 hr at 39°. Results are avg of duplicate flasks. Serum dialysed for 48 hr against bicarbonate buffer. Gas phase: 5% CO_2 - 95% O_2 in serum and Krebs-bicarbonate buffer, 100% O_2 in Krebs phosphate buffer. Albumin screened 3 days for rat, 4 days for rabbit. Volume of iodoalbumins 0.2 ml in serum, dialysed against saline. Activity/flask in cpm: Rat, 260,000 unscreened, 32,000 screened; rabbit, 140,000 unscreened, 91,000 screened.

breakdown rates of less than 0.5-3% could not be evaluated reliably.

Results. Tissue and organ slices from rabbits and rats were incubated with their homologous plasma proteins as well as with human iodoalbumin. Slices were prepared from liver, kidney, spleen, lung, heart, small intestine, diaphragm, skeletal muscle and skin. A few experiments were performed with liver homogenates. Twenty experiments were performed employing tissues from about 80 animals. Since all experiments were essentially negative, and very limited or no plasma albumin breakdown was detected, only a few typical experiments are presented.

Exogenous screened and unscreened iodoalbumin. Table I represents experiments on incubation of liver slices and liver homogenates with screened or unscreened homologous iodoalbumin. With unscreened iodoalbumin there was breakdown by rat liver, but very little by rabbit liver preparations. On the other hand there was no significant breakdown in either case with screened iodoalbumin. The results were not markedly affected by composition of medium or by protein concentration, which was 10-20 times higher in serum than in buffers. It is unlikely that liberation of I^{131} of unscreened iodoalbumin by rat liver, independent of albumin concentration, represents true albumin breakdown. It appears then, that screened iodoalbumin is not degraded *in vitro* by rat or rabbit liver. McFarlane(8) first pointed out the distinction in catabolism of screened and unscreened iodoalbumin. This difference is due to presence in unscreened iodoalbumin of fractions of much shorter halflife than the bulk of labeled protein. A marked difference in catabolism of the 2 types of preparations has been reported in perfused liver(3,4).

Extravascular iodoalbumin breakdown. The absence of breakdown of added iodoprotein by tissue slices might be due to inability of exogenous albumin to penetrate into the slice. To determine whether extravascular albumin is broken down in slices, animals were injected with iodoalbumin and after several days tissue slices prepared from organs of these animals. Iodoalbumin content of tissue was presumably due in part to presence of residual blood and in part to extravascular exchangeable albumin. This later fraction may be considered as representing endogenous albumin.

Rats were injected with human iodoalbumin and sacrificed 2-3 days later. Rabbits were injected with homologous iodoalbumin and used 1-2 weeks later. Results are summarized in Tables II and III. In rats, 20-30% of activity in slices was in the non-protein fraction, but in rabbits non-protein I^{131} constituted less than 5% of total activity. From the specific activity of plasma albumin, albumin content of viscera was estimated to range from 1-3 mg/g, and of muscle and skin about 0.5 mg/g. Heterologous injected iodoalbumin is broken down as fast(2) or faster than the homologous compound. From Table II it appears that up to 2% of albumin present in viscera was broken down. This represents a *maximal* breakdown of about 0.01 ± 0.01 mg/g in liver and 0.03 ± 0.03 mg/g in lung in the 3 hour period. This amount of breakdown is of doubtful significance and it is not certain whether any breakdown oc-

TABLE II. Breakdown of Preinjected Human Iodoalbumin by Rat Tissue Slices.

		Exp. 1		Exp. 2		Exp. 3		
		% non-protein I^{131}						
Tissue	Approx. albumin content, mg/g	Initial	Final	Initial	Final	Initial	Final	Avg increase in non-protein, %
Liver	.8	17	19	31	34	37	38	2
Kidney	1.3	32	35	32	30	49	46	–1
Lung	3.2	9	10	6	7	9	9	–1
Spleen	1.1	27	32	30	29			2
Muscle	.5	10	10	10	11	11	10	0

Rats were inj. with about 100 μc of commercial human iodoalbumin (RISA) and sacrificed 2 to 3 days later. 250 mg slices in 2 ml bicarbonate buffer. 95% O_2 - 5% CO_2, 37°, 3 hr. Results are avg of duplicate flasks. Total activity/flask from about 300 (muscle), to 2000 (lung) cpm.

curred at all. Similar results were obtained with homologous rat iodoalbumin. In slices of rabbit viscera the increase in non-protein activity after 3 hour incubation ranged from 0.5-1.5% of total activity. There was no increase in muscle (Table II). Maximal breakdown is of the same order as in rat. Again, the values are of doubtful significance, since such low rates of breakdown cannot be measured reliably by our methods.

S^{35} labeled plasma proteins. Catabolism of S^{35} biosynthetically labeled rat and rabbit plasma albumin and globulin by a variety of tissue slices was also studied. The only tissue that broke down S^{35} labeled plasma proteins at an appreciable rate was intestinal mucosa. This breakdown is likely to be due to presence of digestive enzymes in this tissue. As results with other organs were similar to those obtained with iodoalbumin and essentially negative, the data are not presented here in detail. Also a small amount of S^{35} amino acids liberated from albumin breakdown may be reutilized for protein synthesis. Reutilization of liberated I^{131} on the other hand, does not occur.

Discussion. In vivo experiments indicate that liver is a major site of plasma albumin breakdown(2). Our results, in which slices of small intestine were the only tissue to catabolize exogenous plasma protein seem to be inconsistent with *in vivo* results. Failure to observe significant breakdown in organ slices is not likely to be due to lack of penetration into the slice. When iodoalbumin was injected so that tissue extra-vascular albumin became labeled, no significant breakdown was detected (Tables II and III). Breakdown of screened iodoprotein also did not occur in liver homogenates.

Minimal value for plasma albumin turnover in the rat is estimated to be about 2.5 mg/hr/

TABLE III. Breakdown of Preinjected Iodoalbumin by Rabbit Tissue.

		Days after inj. of albumin				
		7		14		
		% non-protein				
Tissue	Approx. albumin content, mg/g	Initial	Final	Initial	Final	Avg increase in non-protein, %
Liver	.8	2.4	2.9	2.8	3.4	.5
Kidney	3.5	3.6	4.2	3.0	2.9	.4
Lung	3.0	1.7	3.2	2.7	3.0	.9
Spleen	1.6			2.8	3.3	.5
Small intestine	1.2	2.6	3.8	1.4	1.7	.8
Heart	2.8			1.3	1.3	
Diaphragm	.7			1.6	1.4	–.2
Liver homogenate		2.2	2.4	2.4	2.7	.2
Kidney "		3.1	3.3			.2

0.5 g rabbit tissue slices (spleen 0.25 g) incubated with 3 ml Krebs Ringer bicarbonate buffer. Gas phase: 95% O_2 - 5% CO_2, incubated at 37° for 3 hr. Homogenate 1:1 in 0.25 M sucrose, 1 ml incubated with 2 ml buffer. Results are avg of duplicate flasks. Total counts ranged from 1400 cpm vessel (diaphragm) to about 7000 (lung, kidney) cpm.

100 g body weight.[*] Assuming that ⅔ of the breakdown occurs in rat liver, we obtain a breakdown rate of 0.4 mg/hr/g liver. In rabbit the corresponding breakdown would be 1.0 mg/hr 100 g body and 0.15 mg/hr/g liver (1). Breakdown rates of this order could be readily detected in our experiments since the lower limit of methods used would reveal breakdown of at least 0.01 mg/hr of exogenous albumin/g of tissue.

The only detailed report of plasma protein breakdown in liver slices appears to be that by Roberts and Kelley(6). They found that when plasma proteins, labeled biosynthetically with phenylalanine 3-C^{14}, were incubated with liver slices, incorporation of C^{14} into CO_2, glucose, and glycogen occurred. Their paper reveals certain inconsistencies in results. Rate of albumin breakdown, as well as production of respiratory CO_2 implied by their results seems unphysiologically high. Thus, a conservative estimate of albumin breakdown, computed from their data, is 11 mg/hr/g liver.[†] Even this low estimate is of a different order of magnitude than that occurring *in vivo*. The presence of a contaminant of high specific activity in their plasma protein preparations may account for such results.

In biosynthesis of plasma proteins from S^{35} and C^{14} precursors, non-protein radioactive compounds, tightly bound to protein may be formed. Also non-protein compounds, absorbed on protein may be labeled during iodination with I^{131}. In short term experiments, as with slices or organ perfusions, liberation of small amounts of activity may erroneously indicate appreciable degradation rates of plasma proteins. Since this activity may be derived from bound non-protein compounds great caution in interpretation of such results is required.

Recently abstracts reporting plasma albumin breakdown by liver slices(11), and liver mitochondria have appeared(12). Since the preliminary reports lack adequate experimental detail, these findings cannot be evaluated. Armstrong and Tarver(13) on the other hand, could not detect $C^{14}O_2$ formation from C^{14} labeled plasma albumin by rat liver slices.

To demonstrate *in vitro* breakdown, special conditions, as yet unknown, may be required. Therefore no final conclusions on the role of tissues in plasma protein catabolism can be derived from negative results in tissue slice experiments as obtained above.

Summary. I^{131} labeled human albumin and screened and unscreened iodinated rat and rabbit albumins were incubated with tissue slices, prepared from rat and rabbit liver, kidney, spleen, lung, heart, muscle, and skin. No breakdown was observed in rat and rabbit liver homogenate. When iodoalbumins were preinjected into animals prior to preparation

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[*] Taking plasma volume as 4% of body weight, plasma albumin concentration as 30 mg/ml, total body albumin pool 2.5 as large as plasma pool, and a turnover time of rat plasma albumin of 120 hours (about 80 hour halflife) we get $\frac{30 \times 4 \times 2.5}{120} = 2.5$ mg/hr/100 g rat. Liver is assumed to constitute 4% of body weight. In rabbit albumin content is about the same but turnover time is taken as 300 hours.

[†] Assuming that 25% of carbon 3 of phenylalanine liberated from protein is catabolised to CO_2, and using Roberts *et al.*(6) data in Table II (100 mg slices, 10 mg of albumin, 2¼ hr incubation, 5.7% $C^{14}O_2$) we calculate that $\frac{5.7 \times 10 \times 100}{100 \times 2.25 \times 25 \times 0.10} =$ 11 mg/g/hr were catabolised by liver slices. The assumption of 25% yield of CO_2 from carbon 3 of phenylalanine is likely to be far too high and the albumin breakdown much higher than the minimal value computed here. We found that when 3-C^{14} phenylalanine was incubated with liver slices the main water soluble metabolic products were unidentified phenolic acids and appreciable amounts of activity were incorporated into tissue proteins. CO_2 yield was low, less than 10% of catabolised C^{14}. Assuming also that CO_2 yield from all carbons of protein is the same as from carbon 3 of phenylalanine and taking albumin to consist of 55% of C and using Roberts data from his Table II we obtain $\frac{5.7 \times 10 \times 0.55}{100 \times 2.25 \times 10 \times 0.014} =$ 100 μmoles of respired CO_2/g/hr. No doubt however, additional CO_2 is derived from endogenous substrate, and also the average CO_2 yield from protein carbon is much higher than that from phenylalanine. Thus this calculation certainly gives a minimal value and the true value as calculated from Roberts $C^{14}O_2$ yields should be much higher. Roberts himself determined directly respiratory CO_2 production by his liver slices, and the maximal value observed by him was 69 μmoles of CO_2/g/hr.

of slices, to introduce iodoalbumin into the extravascular exchangeable pool of various organs, significant breakdown did not occur. Breakdown of exogenous S^{35} biosynthetically labeled homologous albumin and globulin occurred only in slices of small intestine.

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Incorporation of P^{32} into the O-Phosphodiester of L-Serine and Ethanolamine in the Turtle.* (25679)

PADMASINI AYENGAR AND EUGENE ROBERTS
Dept. of Biochemistry, Medical Research Inst., City of Hope Medical Center, Duarte, Calif.

Roberts and Lowe(1) reported presence of O-phosphodiester of L-serine and ethanolamine ([2-aminoethyl]-[L-2-amino-2-carboxyethyl]-phosphoric acid) in turtle tissue. This compound (SEP) also has been found in tissues of snake and alligator. SEP and the DL-serine phosphodiester have been synthesized (2). A similar compound and a phosphagen thereof have been found in the earthworm in which the 2-aminoethyl group is replaced by 2-guanidoethyl or phosphoguanidoethyl group (3). The purpose of our study was to obtain information on biosynthesis of SEP in turtle tissue. A preliminary report of this work has been made(4).

Results. Comparison of isolated and synthetic SEP. Mobilities of isolated SEP(1) and the synthetic compound(2)† were compared in 4 solvent systems by descending chromatography: (a) phenol saturated with water; (b) methyl Cellosolve-water (9:1); (c) acetone-acetic acid-water (4:2:2); and (d) n-butanol-acetic acid-water (8:20:20). The compounds were visualized by spraying with ninhydrin. The results are shown in Fig. 1. In each solvent the compound on the left was the isolated material, that on the right the synthetic material, and in the middle a mixture of the two. Approximately 200 γ of each were used. In each case the natural and synthetic materials showed identical mobilities.

Incorporation of P^{32} orthophosphate into SEP. After drilling holes through plastron above the heart with a trephine, approximately 1 mc of P^{32} orthophosphate in isotonic saline was injected into the ventricle of each of 5 unanesthetized turtles (*Pseudemys elegans*), which were sacrificed at 9, 26, 48, 76 and 99 hours, respectively, after injections. The holes were covered with adhesive tape after injection to prevent contamination and leakage of body fluids. The various organs were removed and homogenized in 10 volumes of 80% ethyl alcohol. After centrifugation the clear supernatant fluids were evaporated to dryness under stream of nitrogen. Residues were taken up in 50% alcohol so that 0.1 ml of the mixture was equivalent to 100 mg of original fresh weight of tissue, and 0.1 ml aliquots were spotted on paper for chromatography. Two-dimensional chromatograms were run routinely in phenol-water and

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† Kindly supplied by Prof. David Lipkin.

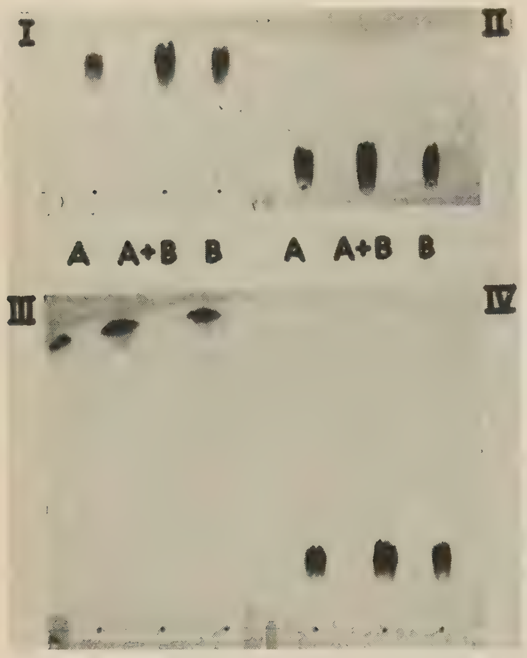

FIG. 1. Mobilities of isolated (A) and synthetic (B) SEP in phenol saturated with water, I; methyl Cellosolve - water (9:1), II; acetone - acetic acid - water (4:2:2), III; and n-butanol - acetic acid - water (8:20:20), IV.

lutidine-water or methyl Cellosolve-water systems. Although use of methyl Cellosolve for the second dimension is in some regards inferior to lutidine, it permits separation of SEP from ethanolaminephosphate (EP), which the lutidine system does not accomplish. After chromatography the sheets were placed in contact with Type K industrial X-ray film for 7 days and were then sprayed with 0.2% ninhydrin in butanol. Photographs were made of both chromatograms and radioautographs. Tissues studied in this manner included liver, kidney, heart, brain, pancreas, sacculus rotundus (a glandular tissue located near the pancreas), and white and red muscle.

The results of ninhydrin sprayed chromatograms in Fig. 2, 4, and 6 illustrate previously observed finding(5) that patterns of free or easily extractable amino acids in extracts of tissues of a particular species differ from each other. The same tissues from different turtles showed somewhat greater variability than is found in mammalian or avian species. The highest concentrations of SEP were found in extracts of red (Fig. 2) and white muscle and heart (Fig. 4), which tissues also contained highest levels of taurine. Smaller amounts of SEP were found in other tissues. In all tissues only small quantities of EP were detected.

Incorporation of P^{32} took place into a number of constituents detectable on radioautographs. However, attention will be drawn

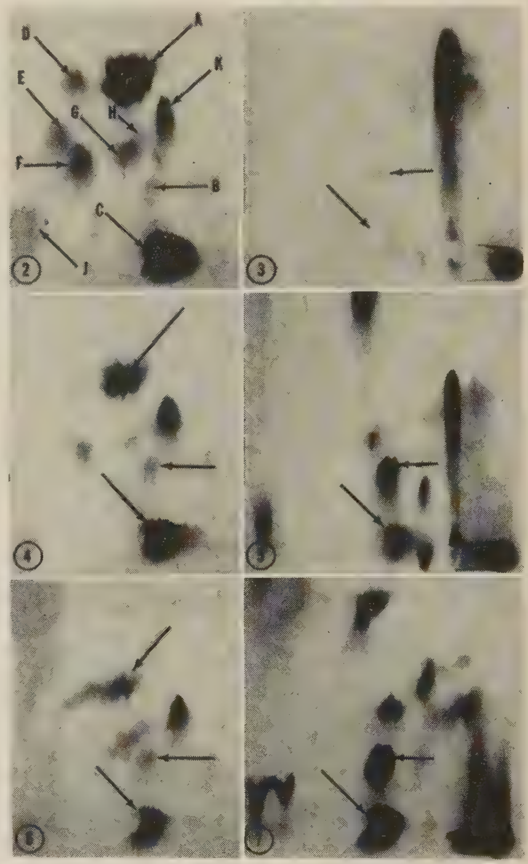

FIG. 2, 4, and 6. Paper chromatograms of extracts obtained from 100 mg of original fresh wt of red muscle, heart, and kidney, respectively, from a turtle 99 hr after administration of approximately 1 mc of P^{32}.

FIG. 3, 5, and 7. Corresponding radioautographs. The solvent directions on chromatograms are phenol - water from right to left and methyl Cellosolve - water from bottom to top.

Constituents identified on Fig. 2 are: Taurine, A; ethanolamine-phosphate (EP), B; phosphodiester of serine and ethanolamine (SEP), C; alanine, D; glycerylphosphorylethanolamine, E; glutamine, F; glycine, G; serine, H; arginine, J; glutamic acid, K. On all subsequent chromatograms arrows point to taurine, EP, and SEP and on the radioautographs to EP and SEP, the direction and position of arrows clearly identifying these constituents.

only to SEP and EP. Kidney (Fig. 6 and 7) and heart (Fig. 4 and 5), which showed highest degree of labeling of SEP, also had the greatest radioactivity in EP. Liver, heart (Fig. 4 and 5), and kidney (Fig. 6 and 7), showed greatest number of P^{32}-labeled constituents on the radioautographs. In all instances visible labeling of EP occurred before that of SEP. Under conditions employed kidney, liver, heart, and brain showed detectable P^{32} labeling in EP at 9th hour after injection of P^{32} orthophosphate, while autographs showed detectable radioactivity in SEP only at 26, 48, 76, and 76 hours, respectively, in the same extracts. The pancreas, sacculus rotundus, red muscle, and white muscle showed detectable label in EP at 26, 48, 48, and 48 hours, respectively, while only in the 99 hour sample was there any radioautographic evidence of labeling of SEP in these tissues. While the quantity of EP detectable on chromatograms was less than that of SEP in all tissues, the extent of labeling of EP was approximately the same as that of SEP in kidney and heart and greater in other tissues, indicating the much higher specific activity of EP. The results did not lead to identification of any P^{32} containing compounds other than EP which might be implicated as a possible precursor of SEP. It is interesting that red (Fig. 2 and 3) and white muscle, which contained the largest quantities of extractable SEP, showed least radioactivity in SEP and EP and the smallest number of labeled constituents on radioautographs.

Streak preparations of pooled extracts from tissues of the 99 hour turtle were made on 10 large sheets of paper, extract equivalent to 1 g being placed on each sheet, and were run one-dimensionally in methyl Cellosolve. Marker strips on the edge were sprayed with ninhydrin and the SEP band was outlined and eluted with water, evaporated to dryness under a fan and lamp. The dried eluate was taken up in 0.9 ml of water and 0.3 ml were employed for preparation of the DNP derivative. Chromatography in dioxane-ammonia solvent (4:1) separated the DNP derivatives of synthetic serine phosphate, ethanolamine phosphate, and SEP. It was found upon radioautography of the chromatograms of the isolated material that radioactive areas corresponded exactly with the DNP derivative of SEP on the chromatograms, thus showing unequivocally that the P^{32} label was located in SEP.

In vitro experiments. *In vitro* experiments with intact beating hearts, kidney and liver slices, brain homogenates, etc., incubated under a variety of conditions did not give unequivocal evidence of incorporation of P^{32} into SEP or of C^{14} from labeled glycine, acetate, or glucose. Only in the incubation of the erythrocytes of turtle blood with P^{32} in plasma was evidence obtained for a small *in vitro* incorporation of the label into SEP after 8 and 12 hours of incubation at 37°. Thus, although present results show that SEP can be synthesized in the turtle, no mechanism for biosynthesis of SEP can be postulated as yet.

Summary. 1. It was shown that intracardially administered P^{32} orthophosphate is incorporated into O-phosphodiester of L-serine and ethanolamine (SEP) and ethanolaminephosphate (EP) in tissues of the turtle. 2. Kidney and heart showed highest degree of labeling of SEP and EP. White and red muscle exhibited smallest uptake of P^{32} into SEP, although containing larger amounts of this compound than other tissues examined. 3. Only incubation of intact erythrocytes in plasma for prolonged periods, of a variety of *in vitro* systems tested, gave any evidence of P^{32} incorporation into SEP.

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Amylase in Fresh Water Fish.* (25680)

Robert L. McGeachin and James W. Debnam, Jr.
Department of Biochemistry, University of Louisville School of Medicine, Louisville, Ky.

Although some information is available on amylases found in certain fish(1,2,3), much of the work was done earlier with rather crude methods then available, with methods now known to be somewhat unreliable, or with methods which gave only qualitative indications(3). Much of this earlier work was done with marine fish rather than fresh water fish. Since we were interested in knowing how amylase in the gastro-intestinal tract and other organs of fish compared quantitatively with mammalian amylase levels(4,5), we investigated amylase in 2 locally available species, the blue-gill and largemouth black bass.

Materials and methods. The fish used were caught in local lakes and kept alive in fresh water from their environment until moment of sacrifice. Any fish which died early as a result of hook injuries was discarded. Blood was obtained by dissecting a flap over the heart and aorta, cutting the aorta and collecting blood in crucible. Gills, heart, liver, gall bladder, kidneys and spleen were removed separately. The gastro-intestinal tract was dissected and washed as previously described (5) and separated into stomach, upper and lower intestine and pyloric caeca. If not analyzed immediately, all tissues were kept in frozen state at –18°C. The tissues were homogenized as previously described(4,5) and amylase contents determined by Van Loon's amyloclastic method(6). The chilled liver tissue for isolated cell preparations was minced with scissors, then placed in Lucite press designed by Dr. R. Duncan Dallam(7), similar to that described by Lata and Reinertson(8). Tissue was gently pressed through 10-mesh (10 to the inch) stainless steel wire screen (20 mm diameter), and washed through with small quantities of cold Ringer's solution. The press was dismantled, cleaned, a 20-mesh screen inserted and a slurry of liver cells and tissue obtained from first operation was pressed through this screen. The process was repeated with screen of 30-, 50- and 60-mesh, obtaining suspension of broken liver cells, intact cells, a few erythrocytes and a very few small clumps of liver cells. This suspension was centrifuged in International Centrifuge, Clinical Model, at 1500 rpm for 15 minutes at 2°, the supernatant discarded, cells resuspended in Ringer's solution and again centrifuged. After 5 or 6 such washings, cells were centrifuged at 3000 rpm for 10 minutes. A small amount of each preparation was dispersed in cold Ringer's solution and examined under microscope. The preparation was usually chiefly whole liver cells with very few clumps, red blood cells or broken cell fragments. If not, the preparation was resuspended, washed and centrifuged again until broken fragments were removed. Overall yields of cells from whole liver were about 10%.

Results. Table I shows that amylase contents of fish tissues other than viscera were lower than serum amylase. There was no indication of high muscle amylase activity previously reported(9). In liver and spleen, amylase levels were well above those of serum, al-

TABLE I. Distribution of Amylase in Various Organs of Bluegill and Black Bass.

	Amylase units/100 ml serum or 100 g tissue	
Organ or tissue	Bluegill	Black bass
Serum	290	870
Liver	4350 ± 550*	1880 ± 190*
Gall bladder bile	3660 ± 420	4370 ± 670
Stomach	1000 ± 110	1290 ± 180
Upper intestine	5740 ±1550	3490 ± 790
Lower "	5970 ±1630	4470 ± 810
Pyloric caeca	7980 ±2210	4680 ±1420
Gill	255 ± 19	
Muscle (back)	108 ± 14	
Heart	280 ± 35	
Kidney	229 ± 15	
Spleen	2900 ± 540	
Ovaries	5840 ± 210 (4)	6560 (2)
Testes	680 ± 60 (4)	450 (2)

All values are avg of 8 determinations for each species except in case of serum, where samples were pooled, and ovaries and testes, where number of samples is given in parentheses.

* Stand. errors of mean.

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though undoubtedly fragments of pancreatic tissue may have caused part of the high levels. Diffuse and disseminated nature of fish pancreas makes it impossible to separate completely the pancreas from whole liver.

Amylase levels of intestinal tract (including pyloric caeca) were well above those of other organs and indicated that the pancreas provides considerable amylase even though the diet of these fish contains very little starch. Amylase *is* found in the gastro-intestinal tract and other visceral organs of the black bass despite previous statements(3) to the contrary.

In studies on isolated liver cells, livers were taken from 35 bluegill. In 7 of these fish, livers were quite pale, almost a light tan color, and extractions with ether indicated that they contained much larger amounts of lipid than normal livers. Since these might represent some pathological condition, these livers were not used in preparation of isolated cells but were pooled and analyzed separately. Their amylase content was 3610 units/100 g tissue compared to 7980 for normal livers. The isolated liver cell amylase (Table II) was much lower than whole liver level but still appreciable, and well above serum amylase, indicating that the liver had some amylase that could not be attributed either to pancreas or blood remaining in liver.

TABLE II. Bluegill Liver Amylase.

Condition of liver	Amylase units/100 g tissue
Whole livers (28)	7980
Isolated liver cells from 28 pooled livers	1390

Amylase in the hepato-pancreas of a carp was 550,000 units/100 g and bile amylase was 23,000.

Summary. Amylase levels of the gastrointestinal tract and other visceral organs of bluegill and largemouth black bass were relatively high, ranging from 1000-8000 units/100 g, considerably above serum levels. Amylase levels of gills, heart, kidney and muscle were relatively low. Amylase was found in isolated liver cells from the bluegill.

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Urinary Excretion of Patent Blue V after Intradermal Injection in Man.[*]

(25681)

SAM A. THREEFOOT

Dept. of Medicine, Tulane University School of Medicine, and Research Lab., Touro Infirmary, New Orleans, La.

Patent Blue V[†] has been used to visualize the lymphatics of man in attempts to understand the role of these vessels in formation of edema of congestive heart failure and other disease states. During these studies, it appeared in the urine(1,2,3) suggesting use of urinary excretion as indicator of lymphatic uptake from interstitial spaces and transport to the venous circulation. Subsequent observations revealed, however, that PBV was absorbed by venous capillaries as well as lym-

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[†] Nat. Aniline Division, Allied Chemical and Dye Corp.

TABLE I. Clinical Data of Subjects Studied.

Subject No.	Age	Sex	Diagnosis and status	Edema	Extremity studied
			Control		
1B	24	♀	Control	Trace-leg	Rt arm
3A (6/23/54)	28	♂	”	0	Lt arm
3B (6/ 6/57)	30	♂	”	0	Rt arm
8	26	♂	”	0	”
10A (12/ 7/54)	34	♂	”	0	Lt arm
10C (5/14/57)	36	♂	”	0	”
12	26	♀	Control—inguinal hernia	0	Rt arm
13	23	♂	Control	0	Lt leg
14	22	♀	”	0	Lt arm
15	32	♀	”	Trace	Rt leg
18	28	♀	”	0-Trace	”
21	22	♂	”	0	”
29	33	♂	”	0	Rt arm
30	22	♀	”	0	”
32	19	♀	”	0	”
34	31	♂	”	0	”
43	26	♀	”	0	”
48	54	♂	Intracranial tumor	0	”
71	22	♂	Control	0	Lt arm
			Congestive heart failure		
9A (11/30/54)	70	♀	Early CHF; untreated; gaining edema	Trace	Rt leg
9B (5/ 8/56)	72	♀	CHF; treated; gradually losing edema	1+	”
16	64	♀	CHF; rapidly losing edema	1+	Lt leg
17	45	♂	Early CHF; untreated	1+	Rt leg
23A (10/ 4/55)	57	♀	Severe CHF; no response to treatment	4+ legs; ascites	”
23B (12/13/55)	57	♀	CHF; slight response to treatment	0 arms	Rt arm
24	64	♀	” no treatment	1-2+	Rt leg
36A (5/29/56)	52	♂	” rapidly losing edema	4+ leg	”
36B (6/ 5/56)	52	♂	” marked improvement	0 arm	Rt arm
45	60	♂	” rapidly losing edema	3+	Rt leg
46	63	♂	” almost compensated	0	”
47	56	♂	Chronic renal disease developing CHF; gaining edema	Trace	Rt arm
49	76	♂	Myocardial infarction; CHF dev'l'ping	Trace	”
			Lymphedema		
7	67	♀	Bilateral inguinal hernia & venous dis.	Trace	Rt leg
19	70	♀	Obstructive lymphedema following radical mastectomy	4+	Lt arm
20	74	♀	Chronic lymphedema of legs; hypochromic microcytic anemia	3+ bilateral non-pitting	Lt leg
31	37	♂	Possible diverticulitis; G.I. neoplasm; edema of undetermined etiology	Trace	Rt leg
39A (6/18/56)	49	♀	Venous disease; immediately after removal of elastic stocking	0	Lt leg
39B (6/25/56)	49	♀	Venous disease; with edema of leg 1 wk after removal of elastic stocking	2+	”
42A (8/13/56)	37	♀	Lymphedema of legs; obesity; varicosities	Non-pitting; 2+ pitting superimposed	”
42B (3/11/58)	38	♀	Lymphedema of legs	Non-pitting; 1+ pitting superimposed	”
54	44	♂	Edema of undetermined etiology of ankles and hands	Slight pitting legs and hands	Rt leg
63A (12/10/57)	22	♀	Unilateral edema of undetermined etiology of right leg	2+ right leg pitting and hard	”
63B (1/ 7/58)	22	♀	Edema of right leg	0 left leg	Lt leg
66	13	♀	Milroy's disease; unilateral left leg	1-2+ unilateral	”

TABLE I (continued)

Subject No.	Age	Sex	Diagnosis and status	Edema	Extremity studied
67	54	♀	Edema of undetermined etiology	2+ pitting bilateral	Rt leg
70	39	♀	Hodgkin's disease; compression of inferior vena cava	1+ pitting	"
74	24	♀	Edema of undetermined etiology; obesity	0	"
77	50	♀	Venous disease; obesity	1+ pitting	"

phatics and thus excretory studies were not indicative of lymphatic function alone but of capillary uptake and transport as well(4).

Materials and methods. Since basic color of each urine specimen differed, it was necessary to develop a spectrophotometric blank by removing the dye from an aliquot of each sample of urine. Addition of 3 ml 5% sodium hypochlorite, 1 ml 20% trichloroacetic acid and 1 ml pH 12.5 buffer to 5 ml urine provided such a "blank," pH 10-11, the spectral characteristics of which were essentially the same as those of urine of the unknown to which buffer only was added. Addition of buffer was necessary for studies which included determination of simultaneous excretion of Patent Blue V and phenolsulfonphthalein. Maximum color of PSP develops at pH 10 and PBV fades rapidly above pH 11. At concentrations greater than 0.5 μg/ml, for determination of PBV or for simultaneous determination of concentration of PSP, excreted rapidly at high concentrations, the error was less than 5%. After intradermal injection of 0.1 ml 11% PBV (11 mg) in arm or leg, all urine was collected as voided in 58 experiments (22 control subjects, 13 with congestive heart failure, 16 with idiopathic or obstructive lymphedema, and 7 with miscellaneous disorders). Collection was continued until dye concentration was too low for measurement. The 48 used in this study are presented in Table I.

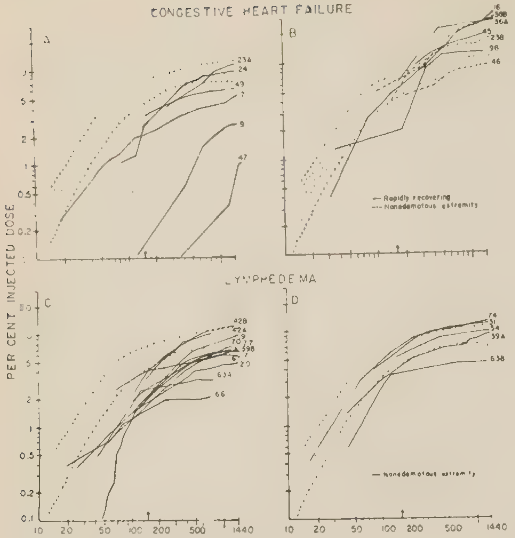
Results. Time of excretion of PBV in urine for 24 hours after intradermal injection is presented in Fig. 1, A, B, C, D. Range of variation in rate of excretion by control subjects was small. Dye appeared in urine in measurable concentration for approximately 24 hours, when approximately 10% of injected dose had been recovered. Patterns of excretion for subjetcs with congestive heart failure varied with stage of formation or loss of edema from the extremity studied (Fig. 1A and B). In subjects with early congestive heart failure developing edema, uptake and excretion of dye are slow and delayed, but in subjects receiving diuretics and losing edema fluid, excretory rates exceeded those of control group, especially after a few hours. In these latter subjects, measurable concentration appeared in urine for several days with as much as 30% of injected dose recovered. The curve of subject 9B (Fig. 1B) responding to treatment and losing edema, fell below the range of control subjects but after some delay exhibited increased output of dye and exceeded that of controls. In general, subjects with lymphedema manifested slower excretion, presumably because of delayed uptake or transport from site of injection. The curve of subject 42 (Fig. 1C) was within control limits for most of its length on 2 separate occasions. Although this subject exhibited some pitting edema (Table I), it was speculated that her disproportionately large legs were due to distribution of fat with no edema producing disease. Subject 74 (Fig. 1D) had large legs with no clinically detectable edema. While the excretory pattern of subject 63B (Fig. 1D) is lower than that of control subjects, it is significantly greater than that obtained when the subject was studied in the edematous extremity (63A, Fig. 1C). This suggests that although there was no detectable edema at the time of study, the patient was beginning to retain fluid and develop edema in that extremity. If the cumulative excretion is plotted for arbitrary time of 150 minutes after injection (indicated by arrow on base line of Fig. 1), differences in these groups are more distinct (Fig. 2A). All subjects studied in an edematous extremity, except those rapidly losing fluid under treatment, had less urinary output of PBV at 150 minutes than did controls.

Since collection of urine was by *ad lib.* voiding only, no accurate estimate could be made of time of appearance of dye in the urine. There was dye in urine of control subjects who voided as early as 10 minutes, but in some subjects with congestive heart failure or lymphedema, urine was collected for 2 hours before dye was evident.

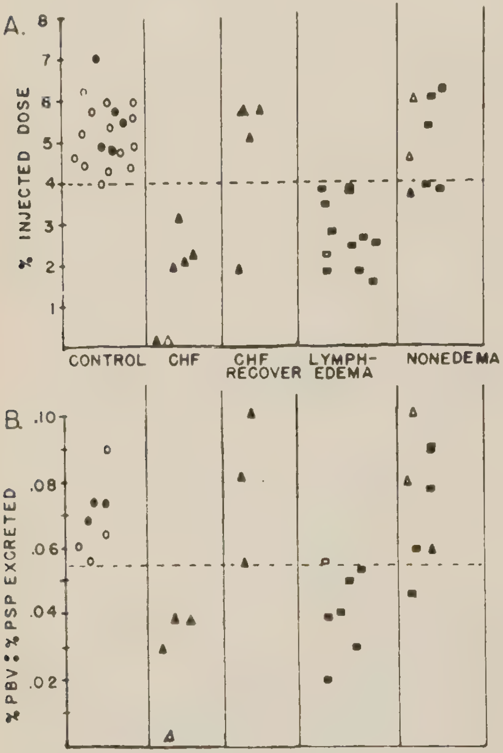
In control subjects, there was no consistent difference between rates of excretion by those subjects injected in arm or leg. In subjects with edema from one cause or another, however, except for subjects diuresing, when a subject was restudied by injection in a nonedematous extremity, there was greater and more rapid excretion.

In consideration of decreased renal function as the cause for decrease in PBV excretion, 1 ml 0.6% PSP (6 mg) was administered simultaneously intravenously and excretion measured as a guide to renal function in 28 subjects. In subjects demonstrating decrease in dye excretion, decrease in PBV excretion was relatively greater than decrease in PSP excretion (Fig. 2B). This decreased PBV: PSP ratio indicates that diminished renal function alone does not explain the delay and decrease in PBV excretion. When equal quantities of PSP and PBV were injected in the same syringe intravenously in a dog, during first 2 hours ratio of PBV to PSP in urine was 0.7 and constant. This indicated that PBV and PSP are probably excreted in similar fashion.

Discussion. One of the original objectives of this study was to evaluate urinary excretion of PBV as indicator of uptake and transport of interstitial fluid by lymphatics. Kin-



① FIG. 1 A-D. Logarithmic plots of time-course of urinary excretion of PBV for first 24 hr after intradermal inj. Large dotted lines demarcate extremes of variation for 19 control subjects. Beginning of each line is at time of collection of first sample. Subjects in 1A and 1C were studied in edematous extremities. Status of subjects presented in Table I. Subject No. at right of each curve. Arrows on base line at 150 min. signify values plotted in Fig. 2A.



② FIG. 2. A. % of inj. dose of PBV excreted by 150 min. after inj. B. Ratio of PBV excretion to PSP excretion at end of 150 min. In 2A and 2B: Circles, controls; triangles, congestive heart failure; squares, lymphedema. Open symbols = inj. in arm; closed symbols = inj. in leg. Dotted line is lower limit for control subjects. Group indicated as NONEDEMA. contains patients with congestive heart failure or lymphedema, as designated by symbols, who were repeated in a nonedematous extremity.

month indicated that this might not be feasible since PBV appeared to be absorbed and transported by other vessels as well(2). In studies of local spread of dye(4) and cannulation of the lymphatics(3), the dyes appeared in blood from venous capillaries and urinary excretion of PBV was not indicative of lymphatic transport alone but of total vascular uptake from an area. There was an inverse relationship between rate and area of spread of the dye at site of injection and rate of urinary excretion. The rates and areas of local spread were greatest in those subjects with edema while urinary excretion was slowest except when they were rapidly losing fluid under treatment. This relationship suggests that decrease in urinary output of dye by these subjects was due, in large measure, to disturbed uptake by either lymphatics or capillaries. A lower PBV:PSP ratio further demonstrated that decreased renal function was not a prime factor in differences observed between subjects with edema and controls, and that diminished uptake by local vessels and/or slower transport to the general circulation were the most reasonable explanations. No correlation between sluggish uptake of PBV and other substances in edema fluid has been attempted, but the implications are apparent.

Summary. Patent Blue V, used to visualize lymphatics is excreted in urine in measurable amounts after intradermal injection of 0.1 ml 11% solution. Differences in rates of excretion between edematous and nonedematous subjects suggested that this may be useful in studying rates of uptake of interstitial substances. Control subjects excreted approximately 10% of injected dose in 24 hours, ½ of which was excreted in the first 2½ hours. Except for patients who were rapidly losing edema under treatment, rates of excretion were less for subjects with edema of various causes. Correlation of urinary excretion of PBV with that of PSP administered simultaneously but intravenously, and correlation of urinary excretion with rates and areas of local spread of intradermally injected dye, indicate that the principal factor in differences in urinary excretion is decreased uptake and transport by vessels at site of injection.

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Effect of Carbon Tetrachloride Upon Glucuronide Formation by Guinea Pig Liver.* (25682)

Kurt J. Isselbacher and Elizabeth A. McCarthy
Dept. of Medicine, Harvard Medical School and Mass. General Hospital, Boston

Glucuronide conjugation is a detoxification mechanism important in metabolism of many endogenous and exogenous compounds possessing alcoholic, phenolic or carboxyl radicals (1). This conjugation occurs primarily in liver and involves a microsomal enzyme (glucuronyl transferase) and uridine diphosphate glucuronic acid (UDPGA)(2,3). Although it seems likely that derangements in glucuronide formation might occur in hepatocellular disease, clinical and laboratory investigations have been contradictory. Both Englert *et al.*(4), and Peterson and associates(5) found no delay in clearance of tetrahydrocortisone (THE) from plasma of patients with liver disease and concluded THE-glucuronide formation was not impaired. In contrast, Cohn and Bondy(6) measured plasma levels of THE-glucuronide in patients with hepatic disease and found only trace quantities of these conjugates, while free THE was present in plasma in significant amounts. Barniville and Misk(7) found a lower urinary glucuronic acid excretion after oral salicylamide adminis-

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TABLE I. Hepatic Glucuronyl Transferase Activity in Normal and CCl_4 Injected Guinea Pigs.

	Liver wt (g)	Glucuronyl transferase activity* Per g liver	Per total liver wt	Per 100 mg microsomal protein
Controls (9)	8.12 ± 1.52	.66 ± .11	5.25 ± .29	2.88 ± .83
Exp. (9)†	9.7 ± 2.0 (p <.1)	.34 ± .08 (p <.01)	3.14 ± .79 (p <.01)	2.75 ± .83
% change from control	+19	−48	−39	

* Activity is expressed in μmoles p-nitrophenol conjugated in 30 min. Results are mean values ± S.D.

† 24 hr after inj. with CCl_4, 0.1 ml/kg.

tration in patients with liver disease. Their study, however, was based solely on urinary excretions so that any derangement in renal function of their patients could have significantly influenced their results. Kunin *et al.* (8) found a decrease in rate of disappearance of chloramphenicol from plasma of patients with hepatocellular disease and suggested that this represented impairment in formation of chloramphenicol glucuronide. Streeten(9) concluded that glucuronide conjugation is not impaired in liver disease. In view of these conflicting reports we studied the glucuronide conjugating capacity and microsomal glucuronyl transferase activity in animals subjected to acute liver injury with carbon tetrachloride. Guinea pigs were selected because of their significant levels of hepatic glucuronyl transferase(3).

Materials and methods. Guinea pigs weighing 200 to 300 g were injected intraperitoneally with carbon tetrachloride (0.1 ml/kg). The animals were then fasted and sacrificed at varying intervals. Normal guinea pigs, fasted for similar periods served as controls. Animals were killed by cervical dislocations and livers perfused with 20 ml of 0.1 M KCl. A 20% liver homogenate (0.1 M KCl) was then prepared with a Potter-Elvehjem homogenizer. The microsomes were isolated by ultracentrifugation(10), and then homogenized in 3 to 5 ml of 0.1 M KCl to yield a suspension with a protein concentration of 30 to 80 mg/ml. Protein measurements were made by turbidimetric technic using Klett colorimeter (11). For studies of glucuronyl transferase activity, p-nitrophenol (Sigma Chemical Co., St. Louis, Mo.) was used as acceptor and its conversion to p-nitrophenol glucuronide measured by decrease in optical density at 400 mμ (3). UDPGA was prepared from UDPG according to Strominger *et al.*(12). The incubation medium consisted of 0.5 μM p-nitrophenol, 0.8 μM UDPGA, 10 μM Mg Cl, 50 μM Tris buffer† pH 7.4 and 0.1 ml microsomal suspension (3 to 8 mg protein) in total volume of 0.75 ml. All incubations were carried out in duplicate for 30 min at 37°C. Three ml of ethanol were then added and aliquots of 0.5 ml removed. After addition of 3 ml of 0.1 N NaOH, optical density at 400 mμ was determined in a Coleman Jr. colorimeter. The micromoles of p-nitrophenol conjugated were calculated on the basis of molecular extinction coefficient of 18 for free p-nitrophenol(3).

Results. Injection of carbon tetrachloride produced changes in gross appearance of livers, apparent as early as 2 hours. Livers instead of their normal brown color had a mottled (brown-gray) appearance, with occasional hemorrhagic areas. Microscopically there was evidence of varying degrees of necrosis and fat infiltration in the centrilobular and midzonal areas, similar to findings described by Myron(13). Cells in the periphery of liver lobule appeared normal both at 2 hours and 24 hours. Activity of glucuronyl transferase 24 hours after carbon tetrachloride injection is shown in Table I. Liver glucuronyl transferase activity was significantly reduced in experimental animals when expressed on weight basis. Thus there was a 48% decrease in activity/g of liver and 39% decrease on the basis of total liver weight. The difference between these 2 values is due to slight increase in liver weight of CCl_4 treated animals. Diminution in hepatic activity of the enzyme could be demonstrated earlier than 24 hours. A reduction in enzyme activity of 19% was observed within 2 hours and as much as 25% within 6 hours after injection of CCl_4. It should be noted in Table I (column 6) that glucuronyl transferase activity of isolated microsomes themselves was not diminished after CCl_4 treatment. The observed reduction in hepatic glucuronide conjugation capacity was therefore due to decrease in amount of microsomes that could be isolated by ultracentrifugation from liver homogenates of CCl_4 treated animals.

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† Tris = tris (hydroxymethyl) aminomethane.

Discussion. These results indicate that acute liver damage by CCl_4 produced a significant reduction in total hepatic glucuronide conjugation as measured *in vitro*. Of interest is the observation that glucuronyl transferase activity in microsomes isolated from damaged liver tissue was not diminished. The histochemical experiments of Myron are pertinent in this connection(13). He observed that activity of succinic dehydrogenase, another particulate enzyme, was not diminished in cells peripheral to midzonal area of liver lobule. The present experiments also suggest that reduction in enzyme activity was on a quantitative basis and did not represent a qualitative alteration of all liver cell microsomes. Electron microscopic data will be needed to resolve this matter definitively. It is likely that structural and functional microsomal alterations may occur in clinical forms of liver disease and thus contribute further to a reduction in hepatic conjugation capacity.

Use of a phenolic substrate such as p-nitrophenol may not reflect ability of microsomes to form glucuronides of other acceptors with alcoholic, phenolic or carboxyl groups. However, to date, no conclusive evidence for more than one glucuronyl transferase has been obtained. Thus, Gunn rats with congenital deficiency of glucuronyl transferase had impairments in formation of glucuronides of such different substrates as o-aminophenol, o-aminobenzoate, menthol, bilirubin(14) and 4-methyl umbelliferone(15). Chojecki and Kern(16) also reported *in vitro* reduction of hepatic glucuronide conjugation in rats with dietary cirrhosis (protein deficiency plus alcohol) and CCl_4 poisoning. However their studies were carried out with whole liver homogenates and no comment was made on activity of isolated microsomes.

That patients with liver disease usually have elevated levels of conjugated bilirubin in serum has often been considered as evidence that hepatic glucuronide conjugation is not impaired, even in severe hepato-cellular failure. It should be recalled however, that non-glucuronide conjugates of bilirubin also occur (17) and contribute to the elevated bilirubin levels under these conditions. Furthermore, in presence of liver disease, hepatic excretory mechanisms are usually significantly reduced and impairment in hepatic excretion of conjugated bilirubin will tend to obscure a lesser interference in glucuronide formation. Under these conditions the bilirubin glucuronide formed, although this be at a reduced rate, would not be effectively excreted into the bile and allow entry of the pigment into the blood stream. Finally, it may be of significance that organs such as kidney, stomach and intestine also have glucuronyl transferase and it is possible, as suggested by Bollman(18), that these tissues contribute to total glucuronide conjugating capacity of the organism in presence of liver disease.

Summary. Intraperitoneal injection of CCl_4 causes reduction in hepatic glucuronide conjugating capacity of the guinea pig as measured by p-nitrophenol glucuronide formation *in vitro*. This reduction is apparent in 24 hours but is demonstrable as early as 2 hours after injection. Glucuronyl transferase activity of isolated microsomes however is not diminished. Therefore, reduction in hepatic glucuronide conjugation induced by CCl_4 is due to a quantitative change in microsomal transferase activity rather than to a qualitative one.

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Placenta and Salivary Gland Fluoride Transfer in the Rat.* (25683)

WOLFGANG BUTTNER† AND JOSEPH C. MUHLER
Dept. of Biochemistry, Indiana University Medical Center, Indianapolis

Concentration of fluoride in drinking water of a pregnant rat must be approximately 10 ppm before an appreciable placenta transfer of fluoride occurs(1). In these studies fluoride transfer occurred by comparing fluoride concentration in pups whose mothers received varying levels of dietary fluoride with comparable controls receiving no fluoride. Such a technic provides for no biological estimation of effect of fluoride. It is possible that even micro-quantities of fluoride transferred through the placenta could result in some anticariogenic effects. For example, Jenkins(2) demonstrated that concentrations of fluoride as low as 1 ppm have a detectable inhibitory effect on salivary acid production. Therefore, to estimate one effect of fluoride, namely, its antisolubility, pregnant rats were given fluoride at different gestation and lactation periods and solubility of pups' molar teeth evaluated for acid solubility.

Materials and methods. Eight pregnant Sprague-Dawley strain rats divided into 4 groups were housed in pairs in raised screen cages in air-conditioned room. Fluoride-free filter paper was placed in each cage for the mother to make nests for her pups. Group I was composed of 2 pregnant rats which served as controls and received fluoride-free drinking water. Pregnant rats in Group II received 50 ppm F (as NaF) in drinking water during pregnancy. One day before delivery she was placed in separate cage, and upon delivery she and her pups were placed on fluoride-free drinking water. Group III was composed of pregnant rats which received the same concentration of fluoridated drinking water as animals in Group II, except that they received fluoride both during pregnancy and lactation, while rats in Group IV received same concentration of fluoride during lactation. All animals received the same stock non-cariogenic diet (F = 0.01 ppm). All pups were sacrificed at 30 days of age and their mandibular teeth prepared for estimation of acid solubility by methods previously described(3). Due to high concentration of fluoride used, it was possible that some fluoride could pass into the oral cavity from salivary tissues and cause a reduction in acid solubility to molar teeth. Since this effect could not be divorced from the antisolubility effect resulting from conversion by blood fluoride of a portion of the hydroxyapatite in developing rat molars to fluorapatite, a further study was planned to investigate the effect of fluoride availability in

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† Present address: University of Mainz, Germany.

TABLE I. Acid Solubility of Mandibular Teeth of Rats Whose Mothers Received 50 ppm F/Day in Drinking Water during Various Periods of Pregnancy and Lactation.

Group	No. of pups	Mean P dissolved in 20 min. (μg)
Control	9	63 ± 3*
F during pregnancy	8	57 ± 2
F during pregnancy and lactation	8	56 ± 3
F during lactation	6	61 ± 2

* Stand. dev.

TABLE II. Comparison of Acid Solubility of Rat Molar Teeth Placed in Various Solutions.

Group	pH of solution	μg P removed	"P"*
Distilled water	5.8	54 ± 4†	
Saliva only	8.0	59 ± 7	
Saliva from rats receiving 2 mg F/day	8.0	55 ± 5	
Saliva to which 400 ppm F was added	8.0	41 ± 3	.02
Aqueous fluoride (400 ppm)	5.5	39 ± 3	.01

* Calculated by means of Student "t" test.
† Stand. dev.

saliva. Intact embedded molar rat teeth were placed in various aqueous fluoride solutions or saliva solutions to which fluoride had been added, and their acid solubility subsequently evaluated. In this study there were 5 groups. Group I evaluated effect of rat molar solubility in aqueous solution of distilled water and served as controls. Group II received distilled water and Group III received 2 mg F/day (as NaF) for 2 weeks. Group IV consisted of evaluation of the effect of rat saliva to which 400 ppm of F (as NaF) was added and Group V compared the effect of 400 ppm F (as sodium fluoride) as an aqueous solution. In Groups II and III, 2 ml of saliva were collected according to technics previously described(4) into which molar rat teeth were placed. Estimations of enamel solubility were made by placing hemi-jaws prepared for acid solubility determination in respective solutions for 16 hours according to methods previously described(3).

Results are found in Tables I and II. The data in Table I show that solubility of mandibular teeth of rats whose mothers received fluoride during entire pregnancy, at a level known to pass the placenta (Group II) was 57 μg P. Similar rats whose mothers received fluoride both during pregnancy and lactation had an acid solubility of 56 μg P, while rats whose mothers received fluoride only during lactation had an acid solubility of 61 μg P. Only the solubility of molar teeth of animals in Groups II and III was significantly different from control group ($P = 0.05$).

The data in Table II show that rat molar teeth placed in distilled water had an acid solubility of 54 μg P. When similar rat molars were placed in saliva 59 μg of P was removed. When saliva from rats which received 2 mg F/day for 14 days (containing approximately 1 ppm F) 55 μg P was removed. If 400 ppm F were added to saliva from control animals, and rat molar solubility evaluated, 41 μg P was removed. This is significantly different from the control group at the 0.02 level of confidence. If an aqueous solution of fluoride at the same concentration was used as in Group IV, 39 μg P was removed. This is also significantly different from the control group ($P = .01$).

Discussion. Micro-concentrations of fluoride passing the placenta contribute significantly to the increased acid solubility of developing molar teeth of rats. These data are of interest to human nutrition since they suggest that mothers residing in a fluoride area may transfer sufficient fluoride to produce a measurable degree of resistance to dental caries in deciduous dentition. Of course, it is not possible to translate reductions in acid solubility in rat molar teeth unequivocally to reductions in human dental caries, but there is sufficient evidence to suggest that increased resistance to acid solubility is one mechanism by which fluoride reduces dental caries. When rats nursed from mothers receiving fluoride both during pregnancy and lactation added antisolubility benefits were not shown. This suggests that availability and biological activity of fluoride may be reduced when present in milk, as shown previously by other technics (5,6). Saliva also either inactivates the biological antisolubility effect of fluoride or under conditions of this study, sufficient fluoride did not pass the salivary barrier to affect solubility of the teeth. It is of considerable interest to learn the reason for these later findings and to determine if salivary glands are selective in their failure to transfer (excrete or secrete) fluoride in contrast to the placenta, as well as to learn more about the biological availability of fluoride in saliva.

Conclusions. When pregnant rats receive 50 ppm F/day during entire pregnancy, their pups' molar teeth show a significant reduction in acid solubility. If fluoride administration is continued during lactation, no added antisolubility effect is shown. Saliva either inactivates fluoride or salivary tissue is selective against fluoride secretion.

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A Hypoglycemic Factor in Leukemic Tumors. (25684)

MURRAY N. SILVERSTEIN, KHALIL G. WAKIM, ROBERT C. BAHN AND EDWIN D. BAYRD
Mayo Clinic and Mayo Fn., Rochester, Minn.*

The syndrome of fibrogenic tumors and hypoglycemia is becoming more frequently recognized(1). Hypoglycemia was observed in rabbits inoculated with a homogenate of spindle cell sarcoma from rats(2); in mice with leukemic tumors(3); and in mice bearing transplantable pituitary tumors(4). We observed earlier considerable amelioration of alloxan diabetes in AKR mice inoculated with lymphatic leukemia tumor BW5147. Comparison of progression of growth of tumor with onset and magnitude of decrease in blood sugar in normal and diabetic animals suggested that increase in the mass of tumor alone did not explain hypoglycemia in these mice. Further studies employing C^{14} glucose revealed no excessive avidity of these tumors for glucose. The pancreas and adrenal glands from normal animals with tumor and from diabetic animals with and without tumor were studied. No islet cell hypertrophy or hyperplasia in normal animals with tumor was observed. The pancreas of tumor-bearing animals and of normal animals revealed equally severe damage to islet cells after administration of alloxan. Furthermore, as diabetes in tumor-bearing animals was ameliorated the pancreas did not show regeneration of islet cells. Adrenal glands of diabetic animals with and without tumor were similar. These findings suggested possible presence of a hypoglycemic factor in this tumor.

Methods. AKR mice and the tumor BW5147 of lymphatic leukemia were obtained from Jackson Memorial Lab., Bar Harbor, Me. Three types of extracts were prepared from 15-day-old tumors. At this age of tumors, mice exhibited profound hypoglycemia and tumors were large with little gross evidence of necrosis. All animals were initially inoculated subcutaneously into the right thigh with 50 mg of tumor extract.† The tumor was homogenized in Waring blendor with 4 ml of 0.9% solution of sodium chloride/g of tumor. The homogenate was refrigerated 48 hours, centrifuged and the supernatant decanted. The fluid was evaporated in a watch glass in air at room temperature to concentration of 1 g of tumor/0.5 ml of saline solution. A second extract was made as described by Scott and Fisher(5) for extraction of insulin. The final material for assay was reconstituted in saline at pH 2.5 so that the material extracted by insulin procedure from 1 g of tumor would be present in 0.5 ml of the acid saline solution. For the third extract the

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* The Mayo Fn., Rochester, Minn., is part of Graduate School, Univ. of Minnesota.

† Silverstein, M. N., Wakim, K. G., Bahn, R. C., Bayrd, E. D., unpublished data.

TABLE I. Saline Extract of Tumor in 8 Mice.*

Blood sugar, mg/100 ml Before inj.	1 hr after inj.	% change
293	283	− 3.4
290	340	+17.2
370	347	− 6.2
453	417	− 7.9
447	420	− 6.0
450	430	− 4.4
445	477	+ 7.2
410	420	+ 2.4
	Mean	− 0.14

* Intrav. dose of 0.5 ml of extract contained 1 g of tumor.

tumor was homogenized with 5 ml acetone (triple distilled in glass)/g. After refrigeration for 48 hours, the mixture was centrifuged 30 minutes, and the supernatant acetone was decanted and transferred to a watch glass for evaporation as before. The material on the watch glass was suspended in 0.9% sodium chloride adjusted to pH 2.5 with hydrochloric acid. Final concentration was so prepared that the acetone-extractable material from 1 g of tumor would be present in 0.5 ml of acid saline solution. Acetone extracts of tumors from animals with alloxan diabetes were compared in potency with similar extracts of tumors from normal animals. Alloxan diabetic AKR mice used as assay animals were lightly anesthetized with nembutal (0.75 μg/20 g mouse given intraperitoneally) and a fresh 1% solution of alloxan monohydrate was administered rapidly by tail vein. Each mouse received 1 mg of alloxan monohydrate/10 g of body weight and was used for assay after 48 hours. For assay the alloxan diabetic mouse was anesthetized with nembutal and 0.12 ml of blood was removed by cardiac puncture. In one group of mice the saline extract was given intravenously slowly (into tail vein) in one dose of 0.5 ml corresponding to 1 g of tumor. In another group the acetone extract was given in same manner, and blood was drawn from heart 1 hour later. Since use of intravenous route caused death, insulin extractable material was injected intraperitoneally. Blood was withdrawn by cardiac puncture either 1 or 2 hours later. True blood sugar was determined by microtechnic on a modified Somogyi filtrate.‡

Results. After administration of saline extract of tumor, the mean change in 8 animals, Table I, was a fall in blood sugar level of 1.13% with ranges from a rise of 17.3% to a fall of 7.9%.

With material obtained by insulin-extraction procedures, the mean change for the 8 animals was a rise in blood sugar, Table II, of 5.5% with ranges from a rise of 22.1% to a fall of 9.3%.

After intravenous administration of acetone extract, all 11 animals showed, Table III, a moderate to severe fall in blood sugar, with mean change of 24.1% with ranges of 19.0% to 30.8% decrease.

TABLE II. Tumor Extract by Insulin-Extraction Procedure in 8 Mice.*

Blood sugar, mg/100 ml Before inj.	1 hr after inj.	% change
460	470	+ 2.2
430	465	+ 8.1
557	680	+22.1
283	287	+ 1.4
530	620†	+17.0
474	430†	− 9.3
500	480†	− 4.0
407	410†	− 0.7
	Mean	+ 4.6

* Intraper. dose of 0.5 ml of extract contained 1 g of tumor.

† Two hr after inj.

The illustration shows time-response curve after administration of acetone extract as described. A fall in blood sugar level of 7% occurred at 15 minutes, of 30% at 30 minutes, of 25% at 1 hour, and of 7% at 2 hours.

TABLE III. Acetone Extract of Tumor in 11 Mice.*

Blood sugar, mg/100 ml Before inj.	1 hr after inj.	% change
620	443	−28.5
520	417	−19.8
603	443	−26.5
543	440	−19.0
447	321	−28.2
467	323	−30.8
447	340	−23.9
490	365	−25.5
489	372	−23.9
540	433	−19.8
523	420	−19.7
	Mean	−24.1

* Intrav. dose of 0.5 ml of extract contained 1 g of tumor.

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‡ Method devised by Dr. Warren F. McGuckin, Sect. of Biochem., Mayo Clinic.

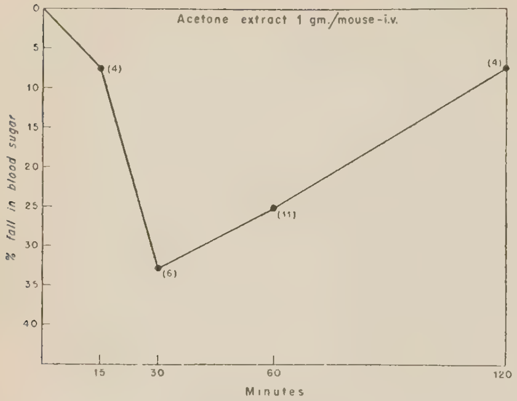

FIG. 1. Time-response curve.

When only acid saline solution at pH 2.5 was injected intravenously in doses of 0.5 ml. a mean rise of blood sugar of 1.5% was observed, Table IV, with ranges of 12.6% rise to an 8.9% fall.

Extracts of tumor obtained from diabetic animals did not produce significantly different hypoglycemia from extracts of tumors of nondiabetic animals. Another interesting finding was the gradual diminution in extract potency with time.

Comment. Simple acetone extracts of lymphatic leukemia tumor BW5147 caused moderate hypoglycemic effects when given intravenously to alloxan diabetic AKR mice. Material obtained by insulin extraction procedures, however, produced negative results by the intraperitoneal route. In human cases of fibrogenic tumors with hypoglycemia, only one tumor produced insulin-like activity(6).

The nature of the hypoglycemic factor in tumors is questioned. Procedures for extraction of insulin indicate that this factor might not be insulin. The question arises, could it be another protein moiety or a lipide? Studies are in progress to determine the nature of the factor.

It would be of interest to determine if many different types of malignant tumors contain an acetone extractable hypoglycemic agent.

Summary. A fall in blood sugar was observed in AKR mice inoculated with leukemic tumor BW5147. Marked amelioration of diabetes resulted from inoculation of alloxanized AKR mice with lymphatic leukemic tumor. Growth of this tumor was studied in normal and diabetic mice. Rate of tumor growth and blood sugar values were correlated. Increase in mass of tumor alone did not seem to explain hypoglycemia in these mice. Consequently, 3 extracts of tumor were made and tested for presence of a hypoglycemic factor. Blood sugar values of diabetic mice were determined before and after injection of tumor extracts. A saline extract did not show a hypoglycemic effect, nor did an extract made by insulin-extraction procedure. An acetone extract, however, exhibited moderate hypoglycemic activity. Time-response curves demonstrated that acetone-extractable material derived from 1 g of fresh tumor produced a fall in blood sugar of 7% at 15 minutes, 30% at 30 minutes, 25% at 1 hour, and 7% at 2 hours.

TABLE IV. Acid Saline Controls in 8 Mice.*

Blood sugar, mg/100 ml Before inj.	1 hr after inj.	% change
550	570	+ 3.6
620	600	− 3.3
364	410	+12.6
358	344	− 3.9
373	340	− 8.9
490	512	+ 4.5
410	407	− 0.7
500	538	+ 7.6
	Mean	+ 1.5

* Dose of 0.5 ml of 0.9% sodium chloride given intrav.

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Fluorescent Treponemal Antibody Test. Modification Based on Quantitation (FTA-200) (25685)

W. E. Deacon, Elizabeth M. Freeman and Ad Harris
Venereal Disease Research Lab., Communicable Disease Center, Chamblee, Ga.

The Fluorescent Treponemal Antibody (FTA) test described previously(1) was critically appraised through participation in the Serology Evaluation and Research Assembly (SERA) Study conducted in 1956-57 (2), and shown to produce highly sensitive and specific results. However, since completion of the SERA Study, a new chemical compound, fluorescein isothiocyanate, has been introduced(3) and is employed to produce labeled antiserums of increased potency(4). Since fluorescein labeled antihuman globulin is the treponemal antibody detector system of the FTA test, an improvement in this reagent is reflected in an apparent increase in test reactivity. This means that although the original FTA test had a desirable level of reactivity, the procedure as performed(5) with presently available reagents has not been so measured. In this study, quantitation of the FTA test was undertaken to determine the present sensitivity and specificity of the test as related to new and improved reagents and to ascertain any changes in qualitative procedure which might be employed to obtain a more satisfactory result.

Materials and methods. Test serums were obtained from the Serum Bank, USPHS Serologic Evaluation Study, and TPI Testing Service, all Venereal Disease Research Lab. Serums from the Serum Bank and TPI Testing service had been preserved by freezing at –20°C. SERA Study serums were selected for testing on basis of categories and availability. Qualitative FTA tests were performed by employing a test serum dilution of 1:5 as described earlier(5) and at dilution of 1:200 (FTA-200). Test serums demonstrating Reactive (R) or Weakly Reactive (WR) results at the 1:5 dilution were retested by preparing serial dilutions and determining a 2+ Reactive endpoint. Results obtained by the experimental qualitative procedure (FTA-200) are expressed in terms of Nonreactive (N) or Reactive (R). Weakly Reactive (WR 1+) findings as designated for the FTA test are recorded as Nonreactive (N) in the FTA-200 procedure. Fluorescein labeled horse antihuman-globulin* was used as the treponemal antibody indicator in all tests. The conjugate was diluted at 1:80 (optimal dilution for this particular lot) in phosphate buffered saline, pH 7.2 containing 2% Tween 80. Lyophilized *Treponema pallidum* antigen was employed for smear preparations. Two smears were prepared for each slide/test serum dilution. Of the 2 smears, the most satisfactory in terms of microscopic appearance (numbers and distribution of *T. pallida*) was used for reading and determining final reactions.

Results. Table I illustrates qualitative results obtained by the FTA-200 test based on selection of a critical dilution factor for test performance. These are compared with results obtained by a group of selected tests performed at Venereal Disease Research Lab and previously reported in the SERA Study(2).

In the Presumed Normal Category, 4 serums demonstrated 1+ readings in the FTA-200 test. Reactive(R) titers of these specimens were shown to be 100 dils.† Other test serums had Reactive (R) titers of less than 100 dils. As previously noted, WR (1+) reactions in the FTA-200 test are considered insignificant and were therefore reported as Nonreactive (N). On this basis, almost complete agreement of FTA-200 test results and the comparative test procedures is obtained.

In Early Syphilis, Untreated, consisting of primary and secondary syphilis, quantitative findings ranged from less than 100 dils (primary group) to 25,600 dils in the secondary category. Lack of agreement by various comparative tests is particularly noticeable in the primary group.

In Early Syphilis, Treated, findings appear

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* Sylvana Chemical Co. Lot No. 201.

† Dils = Dilution of serum tested; *e.g.*, 100 dils = 1:100 dilution.

TABLE I. Comparison of FTA-200 Test and Other Serologic Tests for Syphilis from Selected Clinical Categories of SERA Study.

	FTA quantitation					
	Range	Mode	FTA-200	TPI	Reiter treponeme	VDRL slide
Presumed normals	<5-100	<5	25-N	1-WR; 24-N	25-N	25-N
Primary syphilis, untreated	<100-3200	200	8-R; 2-N	7-R; 3-N	7-R; 2-WR; 1-N	9-R; 1-N
Secondary syphilis, untreated	800-25,600	6400	10-R	9-R; 1-WR	9-R; 1-WR	10-R
Primary syphilis, treated	200-1600	400	4-R	2-R; 2-WR	3-R; 1-N	4-N
Secondary syphilis, treated	800-3200	1600	5-R	4-R; 1-WR	5-R	3-R; 2-N
Late syphilis, treated	200-25,600	1600	28-R	25-R; 3-WR	25-R; 1-WR; 2-N	17-R; 4-WR; 7-N

to be similar to untreated group but with a trend toward lower titers. Again, agreement of comparative tests appears to be somewhat related to FTA titers; *i.e.*, where titers are high agreements are most satisfactory, but when FTA titers are low, comparative tests tend to obtain a variety of results.

In Table II reproducibility and relative reactivity levels of the qualitative FTA (1:5) and FTA-200 tests are compared. Test specimens consisted of aliquots of 20 serums distributed in the USPHS Serologic Evaluation Study. These were sent to 2 separate sections of Venereal Disease Research Lab for testing. Tests were performed by both sections on 40 code-numbered specimens that were 20 serums in duplicate. Results obtained vividly demonstrate the difference in reactivity levels of the 2 procedures. Of most importance is the reproducibility obtained by both sections using the FTA-200 technic on duplicate serums. This is particularly evidenced by agreement of results obtained on the same serum when tested in the 2 sections.

Discussion. Our findings support the view that the FTA test as described in the 1959 Manual of Serologic tests for Syphilis(5) has become considerably more sensitive than the

TABLE II. Reproducibility of FTA-200 by 2 Laboratories on USPHS Serologic Evaluation Study Specimens for August and September 1959.

Reactive pool serum dilutions	Aug. 1959				Sept. 1959			
	Lab 1		Lab 2		Lab 1		Lab 2	
	FTA-200	FTA 1:5	FTA-200	FTA 1:5	FTA-200	FTA 1:5	FTA-200	FTA 1:5
1:2.5	R	R	R	R	R	R	R	R
	R	R	R	R	R	R	R	R
1:5	R	R	R	R	R	R	R	R
	R	R	R	R	R	R	R	R
1:10	R	R	R	R	R	R	R	R
	R	R	R	R	R	R	R	R
1:20	R	R	R	R	R	R	R	R
	R	R	R	R	R	R	R	R
1:40	N	R	N	R	N	R	N	R
	N	R	N	R	N	R	N	R
1:80	N	R	N	R	N	R	N	R
	N	R	N	R	N	R	N	WR
1:160	N	R	N	R	N	R	N	R
	N	R	N	R	N	R	N	R
1:320	N	R	N	R	N	R	N	R
	N	R	N	WR	N	WR	N	WR
1:640	N	R	N	R	N	N	N	”
	N	R	N	WR	N	WR	N	”
N	N	N	N	N	N	R	N	”
	N	N	N	N	N	N	N	”

similar procedure performed in the SERA Study of 1956-57(2). This increase in sensitivity is particularly evidenced by the frequent occurrence of Weakly Reactive (WR) results obtained in the Presumed Normal category of patients (Table I).

Of various factors which have probably played an important part in increased sensitivity of the FTA test, one must certainly consider the introduction and general use of fluorescein isothiocyanate. Other factors may include use of conjugates prepared from higher titered antiserums and incorporation of Tween 80 to enhance antigen-antibody coupling(5). However, regardless of the actual cause of increased sensitivity of the FTA test, it is obvious that this tends to lower the specificity of procedure in terms of syphilis and seriously affects reproducibility of results.

Our quantitative FTA test yielded high titers in secondary and late syphilis. These range from 200 to 25,600 dils. Primary syphilis, on the other hand, demonstrated low titers ranging from 0[‡] to 3200 dils. As has been noted, some specimens in this group produced a 1+ reaction as did some serums obtained from nonsyphilitic donors. If one discounts a 1+ (WR) reaction and reports such reading as Nonreactive (N), as is done in the FTA-200 test, false positive reports are avoided without appreciable loss of ability to detect syphilitic patients. Studies to be reported later indicate that the low grade fluorescence obtained when testing presumably nonsyphilitic patients (1:50 and 1:100) is probably due to treponemal antibodies not associated with syphilis. It should be noted also that in no instance was strong fluorescence or a 2+ Reactive (R) result obtained in the Presumed Normal group at the critical 1:200 dilution.

Summary. 1. A modified FTA test technic designated as FTA-200 is described and recommended on the basis of results obtained by examining well-documented serum specimens from various syphilis categories and a Presumed Normal group. 2. The FTA-200 test possesses a greater degree of reproducibility than the FTA test when results were compared on duplicate serums.

[‡] 0 dils = less than 200 dils in the FTA-200 test.

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Plasma Clearance and Glucuronide Conjugation of 11-Desoxycortisol (Substance S) in Man.* (25686)

Ernest M. Gold (Introduced by Peter H. Forsham)
(With technical assistance of Bernardine Serena and Mary Ann Herron)
Metabolic Unit for Research in Arthritis and Allied Diseases and Dept. of Medicine, University of California School of Medicine, San Francisco

The metabolic fate of a number of adrenocortical steroids in the peripheral circulation of man has been described by Peterson(1). Recently Touchstone *et al.*(2) reported that *11-desoxycortisol* was secreted by the human adrenal in significant quantities, amounting to approximately one-tenth the concentration of *cortisol* in adrenal venous blood. Rate of removal of 11-desoxycortisol from plasma and rate of appearance of its glucuronide-conjugated metabolites in "normal" subjects and in patients with liver disease and hypothyroidism are reported here.

Materials and methods. Patients. Four

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* These studies were carried out under grant from N.C.I. (C2-3995).

patients with absent or suppressed adrenocortical function were studied. Two adrenalectomized subjects were given 1 mg of 9-alpha fluorohydrocortisone[†] and 2 mg of dexamethasone[‡] daily during the study. Adrenal function in the remaining 2 subjects was suppressed by administration of 2 mg of dexamethasone daily. No attempt was made to limit endogenous corticoid secretion in 2 additional patients studied, one with primary hypothyroidism and another with portal cirrhosis and ascites. Each patient was given an infusion of 100 mg of 11-desoxycortisol[§] dissolved in 500 ml of .9% saline intravenously within 30-min. period and heparinized samples of whole blood were collected. The procedure was repeated with each patient on another occasion, substituting cortisol for 11-desoxycortisol. *Measurement of plasma Porter-Silber chromogens (PSC).* Both cortisol and 11-desoxycortisol were estimated as *unconjugated* ("free") PSC in plasma by a modification(3) of the procedure of Peterson *et al.*(4), by which either may be quantitated with equal accuracy. This modification was adapted to the estimation of plasma *glucuronide-conjugated* PSC as follows. Free PSC were extracted from 10 ml aliquots of plasma with dichloromethane in the usual manner(3). The residue was incubated for 18 hr at 45°C with 5000 units of beef liver beta-glucuronidase,[‖] 1 ml of 1 M acetic acid-sodium acetate buffer (pH 4.8) and 1 ml of 1% ethylenediamine tetraacetic acid. A reagent blank consisting of 20 ml of distilled water and 2 μg standards of authentic cortisol and 11-desoxycortisol[¶] dissolved in 20 ml of distilled water was similarly treated. Following incubation, the hydrolysates were extracted with 80 ml of dichloromethane (freshly redistilled). Emulsions were resolved by centrifugation for 5 min at 3000 rpm. The separated dichloromethane extracts were washed and evaporated to dryness; steroid phenylhydrazones were then prepared and quantitated in the usual manner(3).

Results. Following infusions of 11-desoxycortisol and cortisol, plasma PSC concentrations, expressed logarithmically, decreased as a straight line function of time (Fig. 1). Based on rate of removal of PSC from plasma during initial 150 min. after infusions were begun, the biologic half-life of "free" 11-desoxycortisol was less than one-half that of cortisol in "normal" subjects. Beyond 150 min. the average PSC titer after 11-desoxycortisol (at that time 11.2 μg/100 ml) fell off at a much slower rate, the slope approximating that found for cortisol. In contrast to "normal" subjects the patient with liver disease and another with hypothyroidism showed a marked delay in plasma clearance of both 11-desoxycortisol and cortisol (Fig. 2) with disappearance rates of the 2 steroids remaining proportional, that of 11-desoxycortisol being twice as rapid as that of cortisol.

Rate of appearance in plasma of *glucuronide-conjugated* Porter-Silber chromogens after infusion of each of the 2 steroids differed greatly (Fig. 3). After giving 11-desoxycortisol, a peak concentration was achieved rapidly, the mean amounting to 216 μg/100 ml by the end of 30-min infusion period, with a rapid decline thereafter. At completion of cortisol infusions average level of glucuronide-conjugated PSC, which was only 40.6 μg/100 ml, remained essentially unchanged for 150 min. Once peak levels declined, the conjugates of both steroids were removed from plasma at an identical rate, the half-time for each being 102 min.

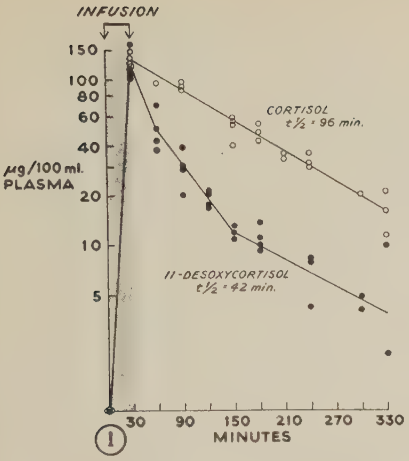
[†] Kindly provided as Florinef® by S. C. Sutton, E. R. Squibb & Sons.

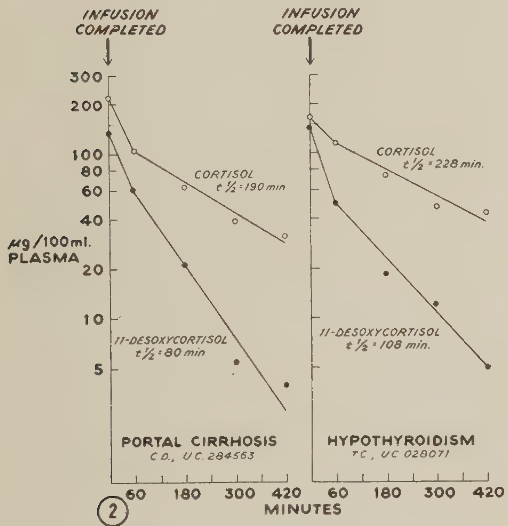
[‡] Supplied as Decadron® through courtesy of Dr. Elmer Alpert, Merck, Sharp & Dohme, West Point, Pa.

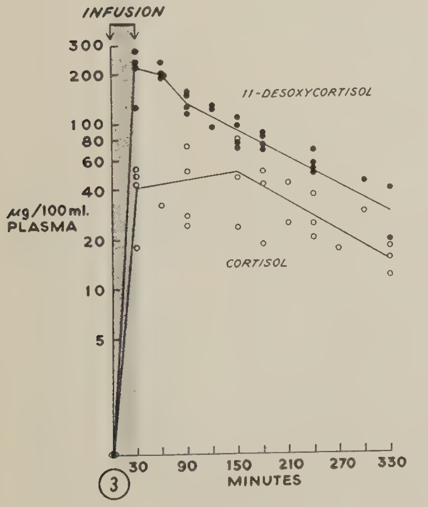
[§] 11-desoxycortisol (delta-4-pregnene-17 alpha, 21-diol, 3,20-dione) was supplied by Dr. Harold Upjohn, Jr., Upjohn Co., Kalamazoo, Mich. Cortisol (free alcohol) (delta 4-pregnene-11-beta, 17 alpha, 21 triol, 3,20-dione) was provided by Dr. Elmer Alpert, Merck, Sharp & Dohme, West Point, Pa.

[‖] Ketodase®, Warner-Chilcott Labs., Morris Plains, N. J.

[¶] Chemically pure cortisol and 11-desoxycortisol standards were supplied by Dr. L. H. Sarett, Merck, Sharp & Dohme, West Point, Pa. For most studies, cortisol was the only standard used since its molar absorbance as a Porter-Silber chromogen is essentially identical to that of 11-desoxycortisol(3). Under conditions used for color development in the present study, tetrahydro-cortisol was essentially equal to cortisol in chromogenicity.







Discussion. Unconjugated 11-desoxycortisol disappears from peripheral circulation twice as rapidly as cortisol. Its removal time, while somewhat shorter than that of corticosterone(5), approaches that found for aldosterone and cortisone(1), suggesting that the longer biologic half-life of cortisol, compared to that of any of these other naturally occurring steroids, is a unique property of the cortisol molecule possibly related to hydroxylation of *both* carbons 11 and 17, which may play a critical role in protecting cortisol from metabolic degradation.

A delay in plasma cortisol clearance has been observed in liver disease(6) and hypothyroidism(7). The present findings indicate that catabolism of 11-desoxycortisol is also impaired, and to the same extent, in these disorders.

Rapid appearance of high titers of glucuronide conjugates after administration of 11-desoxycortisol, in contrast to the more prolonged but lower peak levels after infusion of cortisol, suggests that this difference in removal rate of the *free form* of both steroids is related to appearance rate of their glucuronide metabolites. Existence of independent enzymatic mechanisms for reduction of both 11-desoxycortisol and cortisol, which vary in rate as well as specificity, has been demonstrated in preparations of rat liver. A rate-limiting step occurs prior to conjugation of the molecule with glucuronide and involves saturation of ring A by addition of hydrogen to carbon 5, thereby forming the "dihydro-" metabolite(8). Initial *in vitro* studies with incubates of 11-desoxycortisol and rat liver homogenates suggested that its A-ring reduction was catalyzed solely by a 5-alpha reductase, rather than by a 5-beta reductase as in the case of cortisol(9). More recent work, however, has shown that hydrogen may be added to both steroids in either position, al-

FIG. 1. Disappearance of non-conjugated phenylhydrazine chromogen from plasma following infusion of 100 mg of cortisol and 11-desoxycortisol.

FIG. 2. Delayed disappearance of non-conjugated phenylhydrazine chromogens in portal cirrhosis and hypothyroidism.

FIG. 3. Appearance of glucuronide-conjugated phenylhydrazine chromogens in plasma following infusion of 100 mg of cortisol and 11-desoxycortisol.

though the reaction does require hydrogenases which are steroid-specific(10). Moreover, 11-desoxycortisol is metabolized by such *in vitro* preparations at a "considerably faster" rate than cortisol(11). With respect to 5-alpha reductase activity, rat liver microsomes catalyze 11-desoxycortisol approximately twice as rapidly as cortisol(10), a ratio which agrees well with the relative clearance of the 2 steroids from plasma in the present *in vivo* study in man.

These observations suggest that the more rapid removal of 11-desoxycortisol compared to cortisol from the peripheral circulation should be taken into account in estimating total adrenal secretory response to the 11-beta hydroxylase inhibitor SU-4885(12,13) based on periodic plasma levels of free 11-desoxycortisol.

Summary. The biologic half-life of free 11-desoxycortisol in circulating plasma of man, estimated as Porter-Silber chromogens, was 42 min. This may be prolonged to as much as 80 min. in the presence of liver disease or to 108 min in hypothyroidism. Absence of an 11-beta hydroxyl group accelerates clearance of 11-desoxycortisol from plasma, its removal rate being twice as rapid as that of cortisol. 11-Desoxycortisol is rapidly conjugated to a glucuronide. Concentration of its Porter-Silber chromogen metabolites is 5 times greater than that of cortisol at comparable peak levels; the glucuronide conjugates of both steroids disappear from plasma with a half-time of 102 min.

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Viral Growth and Oncolysis in Krebs 2 Cells as Affected by Substrate.* (25687)

MONROE D. EATON, ANTHONY R. SCALA AND HARRIET C. ROUSE
Dept. of Bacteriology and Immunology, Harvard Medical School, Boston, Mass.

In roller tube suspensions of Krebs 2 cells (K2) infected with influenza virus and with glutamine and lactate as the only substrates high titers of hemagglutinins were obtained, but when glucose was added in place of lactate HA titers were significantly lower(1). The newly formed hemagglutinins remained in or on the cells, and infectivity titers were very low. The K2 ascites tumor has such high virulence that it is possible to quantitate tumor producing capacity of a cell suspension by inoculating mice with serial 10-fold dilutions. Application of this technic to roller tube suspensions of surviving K2 cells has made possible a quantitative study of the relation between substrate, growth of virus, and reduction of tumor producing capacity. Results with influenza and Newcastle disease virus (NDV) will be compared.

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* This work was aided by research grant from Nat. Cancer Inst., U.S.P.H.S., and by funds from Eugene Higgins Trust.

Materials and methods. Sources of tumors and virus strains (PR8, WS, and NDV Cal) have been detailed(1,6). The Francis neurotropic WS strain of influenza was received through the courtesy of Dr. R. R. Wagner(4). The medium consisted of Hanks balanced salt solution (BSS) in 2 different modifications as follows: a. Lactate medium contained, besides inorganic constituents of BSS, 0.1% glutamine, 0.1% sodium lactate, 0.1% bovine albumin, and 0.05% more of sodium phosphate than in the original formula. $NaHCO_3$ was omitted. b. Glucose medium contained BSS plus 0.1% glutamine, 0.1% glucose, 0.1% bovine albumin, additional phosphate as above, and 0.18% $NaHCO_3$. Glutamine and bovine albumin prolong *in vitro* survival of ascites tumor cells(7). Cells obtained from 6 or 7 day tumors in mice were washed twice and suspended in medium to give final cell concentration of about 1.3×10^6/ml. These suspensions were incubated at 35° with virus at a known initial hemagglutinin (HA) concentration (8 to 32 HA units/0.5 ml) for periods of 4 to 72 hours. The roller tubes with caps (tightened) were rotated at 12 rpm. *Titrations of virus* were done by Salk's method for HA, and by chorioallantoic membrane tissue culture for infectivity. Degree of absorption of virus by cells was estimated by comparing HA titers of fluids at 0 hours with those at 4 hours (Table I). Cells were prepared for titration of their viral content by washing twice, then freezing and thawing them in hypotonic BSS as previously described(1). *Tumor producing capacity* of samples of cell suspensions was titrated by inoculating mice intraperitoneally with serial 10-fold dilutions made in plain BSS containing 0.1% bovine albumin. Two mice received 0.2 ml of each dilution. Mice were observed for one month when survivors were sacrificed and examined for presence of solid and ascites tumors.

Results. K2 cells were incubated with influenza virus in lactate or glucose medium. At 4, 24, 48, and 72 hours extracts were made from pooled cells of 4-6 tubes and viral HA and infectivity were titrated. Measurements of tumor-producing capacity were done on suspensions incubated with and without virus.

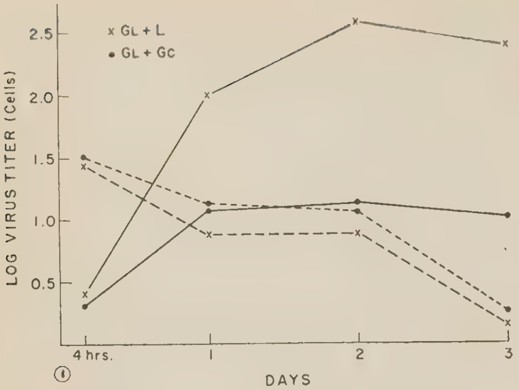
The results of HA and infectivity titrations are presented in Fig. 1. In contrast to results with glucose medium, where maximum titers were 10-15, much more replication occurred in cells incubated with glutamine and lactate. HA titers increased to 300-400 by 48 hours. Infectivity titers of cell material showed no increase in either medium.

It should be emphasized that diminished formation of HA in K2 cells was associated with glutamine, glucose and $NaHCO_3$ combined under aerobic conditions(1). Addition to the medium of either substrate produced significantly higher HA titers than when both were present. The mechanism of this unusual phenomenon is being investigated.

Average results of several measurements of tumor-producing capacity of cells incubated in the 2 kinds of medium, with and without virus, are graphed in Fig. 2. Considering first the results with glucose it will be seen that after 4 hours, virus had reduced the tumor-producing capacity only one log below that of cells incubated without virus. For 3 days, a gradual fall in tumor-producing capacity occurred, but this one log difference from controls remained constant. Increasing amount of virus to 160 HA units resulted in further reduction of tumor-producing capacity.

Addition of PR8 virus to cells suspended in lactate medium caused a marked reduction of tumor-producing capacity as early as 4 hours, and by 3 days the difference from the control was about 3.5 logs. Relative slopes of the 2 curves, virus + lactate and no virus + lactate, suggest that rate of loss of tumor-producing capacity was only slightly augmented between 4 and 48 hours by presence of virus.

Hemagglutinin production in K2 cells by neurotropic and non-neurotropic WS strains of influenza virus and by NDV was also investigated. Results of HA titrations in cells and supernatant fluids from the 2 media are presented in Table I. The WS strains behaved in the same way as PR8 in that much more HA was obtained when lactate was present. This difference is not obvious from HA titers in the fluids except with the FWS strain where a value of 128 was obtained in the lactate medium as compared with 3 in glucose medium.



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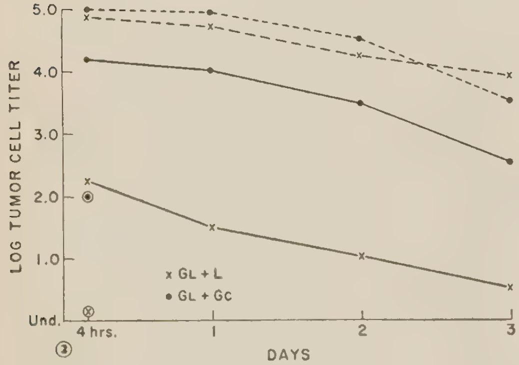

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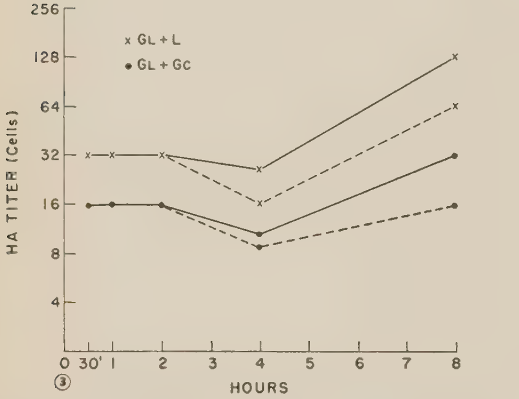

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FIG. 1. Effect of substrate on HA titers (solid lines) and infectivity titers (broken lines) as measured in extracts of washed K2 cells. Dose of virus (PR8) adjusted to give initial HA titer in medium of 1:16. GL = glutamine; L = lactate; Gc = glucose, all at 0.1% concentration. Bovine albumin 0.1% also included.

FIG. 2. Results of titration of tumor producing capacity of K2 cell suspensions incubated under conditions identical with those described for Fig. 1 (solid lines). Broken lines represent control suspensions incubated without virus. Circled points at 4 hr ordinate indicate tumor producing capacity of suspensions incubated with virus at 10 × concentration (160 HA).

FIG. 3. Early HA titers in extracts of washed K2 cells which had been incubated with high concentrations of influenza virus (initial HA fluid 500) in glucose or lactate medium. Broken lines represent result when cells incubated for 2 hr with virus were then centrifuged and resuspended in 0.5% anti-PR8 serum in the same media.

No significant differences in titer were found with NDV in these 2 media and in other experiments using lactate, glucose, or glutamine singly as the sole source of energy results similar to those shown in Table I were obtained.

Since growth of NDV in K2 cells did not seem to be affected by the substrate it was of interest to study its oncolytic activity. At 4 and 24 hours tumor-producing capacity of cell suspensions incubated with virus was 0.5 to 1.5 logs below that of suspensions similarly incubated without virus. At 48 hours, the oncolytic effect was irregular but averaged 2 or 3 logs. Comparison of results with the 2 media did not show differences great enough to indicate an effect of substrate on reduction of tumor-producing capacity by NDV.

Experiments designed to compare rate of absorption of viral HA by cells in lactate and glucose media gave inconclusive results. The data in Table I indicate that between 50% and 75% of the added HA units had disappeared from the fluids by 4 hours and similar results were obtained at ½ and 1 hour. No effect of substrate was observable.

Early measurement of HA titers in cells required an inoculum 20 times as great as that routinely used in previous experiments. After 30 minutes incubation with this amount of virus, cells contained hemagglutinins at titers of 16 to 32. A consistent but slight difference between cells on lactate medium and those on glucose medium was demonstrable (Fig. 3). The increase of HA titer at 8 hours probably represented viral replication.

Hemagglutinins on K2 cells could be reduced to undetectable amounts by treatment with PR8 antiserum. Cells incubated 4 hours with virus were centrifuged and resuspended in the same medium with 5% anti-PR8 rabbit serum (HI titer 1:8000) for an additional 4 hours. After washing these cells, extracts made in the usual manner contained no demonstrable hemagglutinins. With lower concentrations of antiserum partial neutralization of cell-associated HA was observed. Broken

TABLE I. Effect of Substrate on Hemagglutinin Formation in K2 Cells.

	Glutamine + lactate					Glutamine + glucose				
	Fluid			Cells		Fluid			Cells	
Virus	0 hr	4 hr	48 hr	4 hr	48 hr	0 hr	4 hr	48 hr	4 hr	48 hr
WS*	8	2	40	10	320	8	2	16	10‡	40
FWS†	8	2‡	128	10	640	8	2	3	10	20
PR8	24	8	20	4	400	24	12	10	4	15
NDV	8	4	64	4	24	8	8	128	6	24

* Non-neurotropic WS strain of influenza virus. † Francis' neurotropic WS strain.
‡ Trace of hemagglutination at dilution indicated.

lines in Fig. 3 represent effect of 0.5% serum in an experiment similar to that described above. Virus was neutralized to a similar degree in lactate medium and glucose. Replication of cell-associated hemagglutinins occurred on further incubation for 24 hours of suspensions in 0.5% antiserum. Tests for absorption of antibody by uninfected K2 cells were negative.

Discussion. In ascites tumor cells the behavior of influenza virus differs from that of unadapted strains of NDV. Elution of influenza virus at room temperature does not occur(2) and HA formation can be demonstrated both in Ehrlich ascites and K2 cells (1). The studies of Prince and Ginsberg(3, 8) indicate that destruction of Ehrlich ascites cells by NDV is accompanied by an incomplete cycle of virus replication which stops before hemagglutinins are produced. Oncolysis by the neurotropic WS strain of influenza is, however, associated with formation of infectious virus(4).

Incubation of NDV and EA cells in roller tube suspensions at multiplicities similar to those used by Prince and Ginsberg leads to death of cells within 3 days as determined by stainability with eosin and loss of respiration (5). At lower multiplicities used in the present experiments, neither NDV nor the PR8 strain of influenza caused such rapid *in vitro* death of ascites cells as judged by above mentioned criteria(1).

It is apparent (Fig. 2) that events determining growth of virus and fate of cells in mouse peritoneum have already occurred by the 4th hour after mixing K2 cells with influenza virus. After the cells are in the peritoneum their failure to multiply and their destruction are presumably caused by viral replication *in vivo.* Between 4th and 24th hour, when the most active replication of hemagglutinins had occurred, changes in tumor-producing capacity were minor. In lactate medium at 4 hours only 0.1% of cells treated with virus remained capable of producing tumors as compared with 10% in a medium containing glucose and bicarbonate in place of lactate. It appears therefore that in the latter medium fewer cells become infected and that the important factor is adsorption, penetration, or early stage of virus synthesis. Attempts to demonstrate differences in viral adsorption in glucose or lactate media by measuring disappearance of hemagglutinins from the fluid did not yield significant results and only a slight substrate effect was observable when cell-associated HA were measured at ½ to 4 hours. During the first 4 hours complete inhibition of HA in or on the cells by 5% immune serum could be demonstrated. There was, however, no evidence that accessibility of virus to antibody differed significantly in the 2 media. The observation that HA titers actually increased in cells incubated with antibody in excess, suggests that these hemagglutinins were intracellular.

In the absence of convincing evidence that the substrate affects adsorption or penetration of virus we should consider the possibility that an early phase of viral synthesis is involved. As indicated by previous studies with NDV (3) formation of viral antigen demonstrable with fluorescein-labeled antibody may occur without appearance of HA, complement fixing antigen, or infectivity. Possibly it is the formation of this virus antigen or virus precursor *in vitro* which makes the ascites tumor cells incapable of multiplication after inoculation into mice. In glucose medium synthesis of influenza virus precursor would seem, on the basis of this hypothesis, to be less uniformly distributed among individual cells of a suspension than is lactate medium. To explore this idea further studies with fluorescein-labeled antibody are in progress.

Summary. With 3 strains of influenza virus formation of non-infectious cell-associated hemagglutinins in Krebs 2 ascites cells was much greater in a medium containing lactate and glutamine than when glucose was substituted for lactate. Reduction in tumor-producing capacity of cell suspensions by action of influenza virus was correlated with the effect of substrate on HA formation. A marked oncolytic effect was evident in lactate medium at 4 hours, or before the increase in HA. Although Newcastle disease virus was oncolytic at the same concentration as influenza, growth of NDV and its action on tumor-producing capacity were not affected by the substrate.

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Importance of Particle Size in Aerosol Therapy.* (25688)

FRANK W. LOVEJOY, JR., HERBERT CONSTANTINE† AND LUCIEN DAUTREBANDE‡
(Introduced by W. S. McCann)
Depts. of Medicine, Pharmacology and Radiation Biology, University of Rochester School of Med. and Dentistry, and Strong Memorial and Rochester Municipal Hospitals, Rochester, N. Y.

It is known that retention of airborne particulate matter in the respiratory tract is chiefly influenced by size of particles inhaled; the large particles settle in the tracheobronchial tree and smaller ones penetrate deeper into the lungs. When smaller than 0.5 μ in diameter, particles are nearly exclusively deposited upon walls of alveolar ducts and alveolar sacs(1). To emphasize the importance of particle size in administration of medicated aerosols, a small series of experiments was undertaken using 2 different sources of airborne particles: a standard nebulizer (commercially available) producing particles ranging from 0.8 to 7.4 μ, averaging 3.1 μ, according to Grau(2) or from 1.6 to 14.8 μ averaging 6.2 μ according to Barach(3) as measured by optical microscopy and an aerosol generator called D-30, previously described(1) producing particles all below 0.5 μ diameter as measured by electron microscopy. Medicated aerosol was produced from a solution containing 0.1% isoproterenol, 2% phenylephrine in medium composed of equal parts of USP propylene glycol and water. At 5 p.s.i. head pressure the standard nebulizer delivered 9 g of this solution in 10 minutes while the D-30 aerosol generator, during same time and same pressure, delivered 0.3 g only. Four normal subjects (30, 32, 45 and 65 years in age) breathed the medicated dispersates alternately as produced by standard nebulizer and by aerosol generator, duration of inhalation varying from 5 to 20 slow, deep, sub-maximal breaths. Airway resistance was measured according to plethysmographic method of DuBois, Botelho and Comroe(4), and blood pressure, heart rate, and electrocardiogram were recorded in serial fashion.

Results were as follows: 1. Airway resis-

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† Research Fellow, Am. Heart Assn.

‡ Visiting Professor.

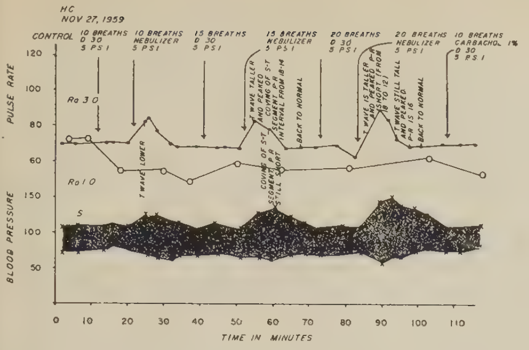

FIG. 1. A representative experiment showing effects of sympathomimetic drug on blood pressure (shaded area), airway resistance (open circles) and heart rate (solid circles). Blood pressure and pulse rate remained stable except after breathing aerosol produced by standard nebulizer.

tance was decreased from control value in every instance on first administration of drug by either standard nebulizer or D-30 generator. Thereafter, it remained below control level. 2. Inhalation of drug dispersed by standard nebulizer always induced changes in heart rate and a rise in blood pressure.

(a) Breathing 5 to 7 breaths of the medicated dispersate from standard nebulizer did not produce subjective symptoms in any subjects, although changes in blood pressure were noticed (Table I, Fig. 1). (b) In 3 out of 4 subjects, rise of blood pressure was accompanied by tachycardia, while in the 4th subject bradycardia was observed during peak of blood pressure elevation (Table I). 3. Every subject noted either a retrosternal dull sensation after 10 breaths of the medicated dispersate from commercial nebulizer or, after 15 breaths, palpitations, retrosternal pain, and/or headache. 4. Inhalation of the aerosol produced by D-30 generator did not cause significant change in parameters under study (Table I, Fig. 1). 5. Following inhalation of 10, 15, or 20 breaths of the medicated dispersate from standard nebulizer, the T wave was either flattened or taller than in control conditions. The ECG remained unchanged while breathing the medicated aerosol from the D-30 generator even after 20 breaths were taken. 6. Duration of changes observed after breathing the medicated dispersate from standard nebulizer was proportional to number of breaths taken. Following 20 breaths, blood pressure changes were noticeable up to 20 minutes after end of inhalation.

Summary. Comparison of effects of 2 aerosols of the same sympathomimetic drug was made. Administration in the usual therapeutic amount of 1 aerosol produced by standard, commercially available, nebulizer caused tachycardia and blood pressure elevation in 4 normal subjects. The other aerosol from a D-30 generator which previously had been shown to relieve bronchoconstriction(5), when breathed in the same manner, failed to have systemic effects. Lack of undesirable side ef-

TABLE I. Individual Values of Blood Pressure (mm Hg), Pulse Rate, and Airway Resistance (cm H_2O/l/sec.) after Inhalation of a Sympathomimetic Aerosol Dispersed by a Commercial Nebulizer or by a D-30 Generator.

			5-10 breaths		15 breaths		20 breaths	
		Control	D-30	Nebulizer	D-30	Nebulizer	D-30	Nebulizer
F.L.	B.P.	120/84	120/84	138/84	126/86	148/88	129/90	148/92
	Pulse	87	88	90	83	100	83	104
	R_A	2.2	1.7	1.7	1.4	1.7	1.3	1.7
H.C.	B.P.	110/75	116/82	124/66	118/70	134/66	112/72	148/66
	Pulse	70	70	82	68	83	71	90
	R_A	2.7	1.8	1.8	2.0	1.8	1.8	2.1
A.S.	B.P.	136/76	138/70	160/70	138/80	184/74	150/84	176/88
	Pulse	91	85	94	85	105	88	106
	R_A	1.2	.5	1.0	.4	.2	.3	.4
L.D.	B.P.	142/84	156/80	196/92	166/86	230/96		
	Pulse	70	65	65	62	54		
	R_A	2.6	1.3	1.4	1.4	1.2		
Avg	B.P.	127/80	133/79	155/78	137/81	174/80		
	Pulse	80	77	83	74	86*		
	R_A	2.1	1.3	1.4	1.3	1.2		

* Note bradycardia in subject L.D.

fects from the latter was presumably due to submicroscopic particles emanating from the D-30 generator. They produced a larger surface area to dose ratio and were able to reach areas in the lung where they are locally most effective even though total dose from generator was 30 times less than from the standard nebulizer for a given number of breaths.

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Chilomastix mesnili Encystment *in vitro*.* (25689)

RUSSELL MICHAEL MCQUAY, JR. (Introduced by I. Davidsohn)
Dept. of Pathology, Mt. Sinai Hospital and Medical Research Fn., Chicago, Ill.

Unlike amebas, which undergo encystment both spontaneously and by inducement *in vitro,* intestinal flagellates seldom exhibited cysts in culture. Although Boeck(1) was the first to cultivate *Chilomastix mesnili* trophozoites in an artificial medium and later other investigators agreed, it was not until 1928 that da Cunha and Muniz(2) observed encystment of this organism in blood agar and N.N.N. mediums. Ball(3), however, failed to confirm their results and also failed to produce cysts in a variety of mediums. In the present investigation 9 stool specimens cultured on a diphasic charcoal medium(4) yielded numerous cysts in addition to trophozoites of *C. mesnili.* They were designated as shown in Table I.

Cyst production was indicated clearly by numerous cysts observed in cultures. Often the cysts were clumped. In 2 instances neither cysts nor trophozoites were seen in stool specimens, but the cultures yielded many trophozoites and cysts.

There was no attempt to isolate any of these strains of *C. mesnili* for further study to determine continuance of cyst production in stock cultures.

TABLE I. Comparison of Stool Findings and Ameba Culture Findings—*C. mesnili.*

Year	Specimen(s)	Stool examination	Cyst findings in culture
1958	3455	No organisms seen	Numerous
1959	1734, 1740*	Trophozoites only	"
	1971, 1972*	*Idem*	"
	2446, 2451*	Many trophozoites, few cysts	"
	3502	Trophozoites only	"
	3677*	*Idem*	"
	3916*	"	"
	6391	No organisms seen	"
	6413	Few cysts only	"

* Purged patients.

Summary. Encystment of *Chilomastix mesnili* was observed in cultures of 9 stool specimens which had been placed in a diphasic charcoal medium.

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Developmental Studies on Emetic Response to Tartar Emetic and Copper Sulfate in the Cat.* (25690)

K. R. Brizzee and K. R. Marshall (Introduced by T. F. Dougherty)
Dept. of Anatomy, University of Utah College of Medicine

In a recent investigation of development of the emetic apparatus in the cat(1), we showed that emetic response first appears in animals weighing between 150 and 250 g (about 3 to 10 days old). Emetic stimulants studied were intravenous lanatoside C (.06 to .80 mg/kg) and total body X-irradiation (12,000 r). While incidence of vomiting with various doses of lanatoside C increased with age of animals, the pattern of response in all positive tests appeared to be much the same at all ages. To provide a more adequate means of comparing sequential chain of events in emetic response in young kittens and adults, and to compare the effects of tartar emetic and copper sulfate with lanatoside C, we carried out developmental studies of emetic effects of the latter drugs with the aid of slow-motion cinematography.

Materials and methods. Kittens and cats varying from newborn to adults were tested with oral copper sulfate in 1% aqueous solution at doses of 8 and 80 mg/kg and oral tartar emetic in 1% aqueous solution at 5 and 50 mg/kg. Over 250 animals were used. Since exact ages of a few of the younger animals were known, the animals were grouped according to following approximate weight means(g): 85, 100, 150, 200, 300, 400, 500, 600, 1000, and 2000 or over. A small amount of red food coloring was mixed with tartar emetic to facilitate recognition of the vomitus. Food was withheld from animals for 15 to 18 hours (over night) before each test, and no test meal was given. Following each test the animal was placed in cage with coarse wire mesh floor and observed 6 to 12 hours or until emetic response occurred. A large sheet of white filter paper was placed beneath each cage. Where no vomiting occurred, the filter paper was inspected for vomitus 24 hours after the test. The chi square method was applied to data from emetic tests to determine whether or not data for the group of animals weighing 150 g and under were significantly different from those of animals weighing 200 g or more for each dose with each drug. Similarly the chi square test was applied to data from groups weighing 200 g or more tested with the low dose of each drug as compared with the high dose. Motion pictures of emetic response in animals representative of the various weight groups were taken by a Bell and Howell model 700H camera on Kodak tri-X film at 64 frames/second. The camera is modified to take a 400 foot magazine and is equipped with electric motor drive.

Results. Results of drug tests are summarized in Table I. Incidence of vomiting with oral copper sulfate at 8 mg/kg (Table I) increased from zero in the first 3 groups to a maximum of about 90% in 600 and 1,000 g animals, then decreased to 66% in animals weighing 2 kg or over. Latency was several hours in the single 200 g animal which responded, and averaged 8 minutes in 6 animals of the 300 g group. Latency increased to around 40 minutes in 400 to 600 g animals then decreased to 32 minutes and 23 minutes respectively in 1 and 2 kg groups. At 80 mg/kg (Table I) incidence was zero in animals of 100 g or less but increased to 100% in all groups above 200 g. Average latency in this series is much shorter than in the 5 mg series, but again the longest average latency was observed in the 500 g group.

With 5 mg/kg tartar emetic (Table I) incidence was zero in all 12 animals of 150 g or less, increased from 50% to 100% between 200 and 400 g, then decreased to final value of 77% in young adults. Latency varied from 7 to 87 minutes with no indication of any trend associated with age. At 50 mg/kg tartar emetic (Table I) incidence was zero in 7 newborn animals of less than 100 g, 25% in the 100 g group and 100% in all older groups.

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* This investigation was supported by research grant from Nat. Inst. Neurol. Dis. and Blindness, and by Univ. of Utah research fund.

TABLE I. Changes in Incidence of Vomiting to Copper Sulfate and Tartar Emetic in a Series of Kittens and Cats Weighing 85 g to over 2 kg.

Wt group, g	No. animals tested	Incidence of vomiting, %	Avg latency, min.	No. animals tested	Incidence of vomiting, %	Avg latency, min.
		$CuSO_4$—8 mg/kg			$CuSO_4$—80 mg/kg	
2000 or more	12	66	23	5	100	8
1000	10	90	32	10	"	6
600	11	91	41	10	"	11
500	11	82	49	5	"	21
400	10	60	39	10	"	10
300	10	60	8	3	"	15
200	10	10	Several hr	8	"	15
150	8	0				
100	4	0		6	0	
85	4	0		5	0	
		Tartar emetic—5 mg/kg			Tartar emetic—50 mg/kg	
2000 or more	9	77	34	4	100	15
1000	6	67	7	10	"	9
600	10	90	40	10	"	11
500	1	100	13	7	"	10
400	10	100	28	10	"	13
300	11	73	87	6	"	42
200	4	50	24	6	"	20
150	4	0				
100	2	0		4	25	98
85	6	0		7	0	

Latency period was appreciably shorter on the average than with the 5 mg/kg dose but showed no clear-cut trends or correlation with age.

Results of chi square tests revealed that differences in incidence between groups (150 g and under; 200 g and over) were highly significant for copper sulfate at 8 mg/kg, and at 80 mg/kg and for tartar emetic at 5 mg/kg and at 50 mg/kg. Also the differences in incidence between high and low doses of copper sulfate for animals of 200 g or over and between high and low doses of tartar emetic were highly significant. All of the above are significant beyond $p = .001$. Differences between successive small groups, as between the 1 kg (90%) and 2 kg (66%) groups with 8 mg/kg copper sulfate are probably not significant.

The sequence of events leading to the emetic act, as determined through direct observation and study of the motion picture films, appeared very much the same in young animals as in adults. It is noteworthy, however, that the reaction in adults is generally much more vigorous than in young animals, especially those representing earliest stages in vomiting. In fact, while vomiting in many adult animals was of a "projectile" type, some of the youngest animals following mild retching merely expelled stomach contents into the mouth. While some variation was observed in events leading to the emetic act in all age groups, the most general sequence observed was as follows: active licking of lips, quick voluntary shaking of head, increased swallowing activity with frequent spasmodic extension of head and neck, yawning, squinting, irregular respiratory movements becoming rapid and fairly regular, a cry, or rather shrill mewing, spasmodic contraction of abdominal wall muscles with simultaneous extension of head and neck, weak at first but becoming stronger and regular with opening of mouth and protrusion of tongue at maximal extension of head. In vomiting it appears that the diaphragm contracts as the initial movement and is followed by contraction of body wall muscles with extension of neck and head, opening of mouth and expulsion of stomach contents.

We noted previously(1) that only a very small percentage of younger animals in which vomiting was not elicited exhibited definite retching movements or movements suggestive of vomiting. In the present study, a number of "negative" animals in the younger groups exhibited enhanced lick-

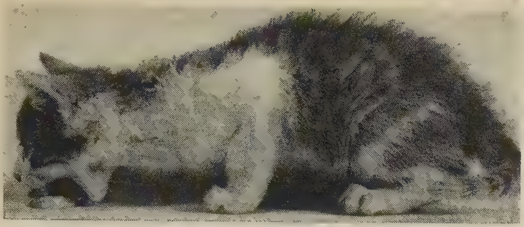

FIG. 1. Typical position assumed by older kittens and cats at time of emetic act. The subject is 600 g kitten responding to 50 mg/kg tartar emetic.

ing of lips, salivation and increased rate of respiration; but definite "retching" movements with coordinated contraction of diaphragm and body wall muscles with extension of head and neck and opening of mouth were observed only in very few such cases. In a few "negative" animals in younger groups, however, it appeared that contraction of diaphragm occurred spasmodically followed by contraction of the body wall muscles without extension of head and neck. These movements were difficult to distinguish from weak efforts at "mewing."

Our previous statement(1) that vomiting occurs only when an animal is in a "standing" position requires some qualification for emesis occurs only when the animal is supporting itself on all 4 feet and is never seen in reclining position or when prostrate. The emetic act generally occurs with the subject in a prone, sitting, or crouching position and in many cases the animal crouches very low as emesis occurs (Fig. 1). The youngest animals exhibiting a positive response (200 to 300 g) often appear to suffer severe malaise and prostration and have difficulty maintaining a crouching position, but invariably make an effort to extend the forelimbs and bring anterior part of body at least into a standing or crouching-sitting position when the emetic act occurs. The single case in which a younger animal (100 g) vomited was unfortunately not observed at the instant of response.

It was observed repeatedly that tartar emetic results in shivering in many animals, especially at higher dose level, a short time after administration of the drug. This reaction was not observed with copper sulfate or in the earlier investigation with lanatoside C.

Comment. Chi square tests showed clearly that the differences in incidence of vomiting in younger groups (85 to 150 g, from about 1 to 3 days of age) as compared with older groups (200 g or over, about 7 days of age or older) were highly significant and that the differences in incidence between lower and higher doses of each drug for animals of 200 g or over were likewise highly significant. Only one out of 22 animals in the younger groups responded to these higher dose levels thus confirming the fact(1) that between 150 and 200 or 250 g stages is a critical period in development of the emetic mechanism in the cat.

Since we obtained different doses by administering different volumes, it might be argued that the results could be explained to some extent on the basis of different volumes or on absolute amount of drug administered. However, at a given dosage on a mg basis at a constant volume, the results would be complicated to a greater extent by considerations of varying drug concentration than by varying volume. We believe the gastric and/or duodenal receptors are more evenly stimulated by varying volume and maintaining concentration than the converse.

That animals in younger groups where incidence was very low or zero (150 g or less) in many cases received a larger amount of fluid as well as a larger absolute amount of drug than animals in older groups where the incidence was fairly high argues against the view that lack of response in these younger animals is due only to the small amount of drug received. For example, the 6 animals in the 100 g group tested with larger dose of copper sulphate each received about 8 mg of drug in .8 cc of fluid (incidence 0) while animals in the 300 g group given the small dose of copper sulphate received about 2.4 mg of drug in .24 cc of fluid (incidence 50%). Thus it appears that neither amount of fluid nor absolute amount of drug can be the determining factor in production of an emetic response. Rather, presence or absence of response depends here on functional maturity of the emetic apparatus itself.

There were no notable differences in premonitory signs or the actual emetic act itself between copper sulfate, tartar emetic, lanatoside C, or total body X-irradiation (except for shivering reaction seen with tartar emetic).

Summary. Incidence of vomiting to copper sulfate (8 mg/kg and 80 mg/kg in 1% solution) and tartar emetic (5 mg/kg and 50 mg/kg in 1% solution) increased significantly as shown by chi square tests, from animals weighing 150 g or less to a group weighing 200 g or more. A significant increase in incidence was also noted between animals (200 g or over) tested with low and high doses of these 2 drugs. While incidence increased to higher dose levels in the latter group no significant increase in incidence to the higher dose levels was found in the younger group (150 g or less). The above findings indicate that the period between 150 g and 200 g stages is a critical period in development of emetic response to copper sulfate and tartar emetic. The emetic act in older animals is more vigorous than in younger groups but the sequential chain of events leading to vomiting is much the same in young and old animals.

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Identification of *Listeria monocytogenes* by the Fluorescent Antibody Technic. (25691)

CHAUNCEY W. SMITH, JOHN D. MARSHALL, JR. AND WARREN C. EVELAND
(Introduced by F. D. Maurer)
Bacteriology, Immunology, and Infectious Disease Branch, Armed Forces Inst. of Pathology, Washington, D.C.

Reports of human infections due to *Listeria monocytogenes* have increased in the past decade, due either to a greater number of such infections or to improved technics and awareness. *Listeria* is associated with many disease syndromes(1-3). Infections due to this organism are generally not distinct clinical entities. King and Seeliger(4) reported, within an 8 year period, 100 isolations of *Listeria monocytogenes* in the United States, only 14 of which have been reported in the literature as case histories. The organism was originally described(5) as a Gram-positive rod with a tendency to palisade formation typical of diphtheroids. Because of this similarity to diphtheroids, the organism is undoubtedly often discarded as a contaminant. More recently it has been reported that pleomorphism is predominant and that the coccoid to diplococcoid form is a more typical property(6). It is easily decolorized, and on blood agar the colonies may resemble streptococci. It is often difficult to isolate, requiring incubation at 4°C for several months(7). Because of increasing importance of this organism in clinical bacteriology and the inherent difficulties in isolating and identifying it, use of the fluorescent antibody technic(8) was investigated to provide a rapid and specific method for its detection and positive identification.

Materials and methods. Somatic antisera. Strains ATCC 7648, Type I (Schultz); ATCC 7644, Type II (Gibson); KC224 (Type III); Seeliger 5214 (Type IVa)(1); and Seeliger 1071/53 (Type IVb) were used to prepare somatic antigens, essentially according to method of Paterson(9). Pairs of mature albino rabbits were injected IV every 4 days with 0.5; 1; 2; 3; 3; and 3 ml of antigen adjusted to a McFarland 3, and bled 5 days after last injection. *Flagellar antisera.* Strains ATCC 7644; ATCC 4428 (Type I); and 5214 were used to prepare the flagellar antigens according to method of Paterson(10). It was found necessary to use an additional inoculum of 1.5 ml of antigen to obtain a good immune response. *Whole cell antisera.* Strains ATCC 4428, ATCC 7644, and 5214 were inoculated in trypticase soy broth and incubated at 37°C for 18 hours. Kolle flasks of trypticase soy agar were inoculated with 0.5 ml of broth culture and incubated at 37°C. The growth was removed with 0.5% formalinized saline and the cells washed 4 times with normal saline. Pairs of albino rabbits were injected IV with 0.1; 0.2; 0.4; 0.8; and 1.0

ml of antigen on successive days. Beginning 10 days later they were each reinoculated with 1 ml on 5 successive days, then bled 7 days after last injection. Prepared whole sera were conjugated with fluorescein isothiocyanate according to Marshall(11). Bovine serum albumin conjugated with Lissamine rhodamine RB 200 was added as counterstain(12). Controls consisted of pooled preimmunization bleedings and an antiserum to *Erysipelothrix insidiosa*(13) both conjugated as above and with the counterstain added. *Preparation of slides. Listeria* organisms were characterized by their biochemical reactions, and motility was determined at 25°C. Stock cultures were maintained on trypticase semisolid medium at 4°C and were transferred every 3 months. All other test organisms were grown and maintained as generally prescribed. Saline suspensions of 18 hour growth at 37°C were placed on clean slides, air-dried and heat-fixed. Smears were overlaid with labeled sera and placed in moist chamber at 37°C for 30 minutes, washed in buffered saline (pH 7.2) for 10 minutes, and mounted in glycerol buffered to pH 7.2. If pH of glycerol is below 7.0, fluorescence tends to be quenched. Duplicate slides were stained with labeled pooled normal rabbit and *E. insidiosa* antiserum. Smears were overlaid with human sera for 30 minutes at 37°C, washed in buffered saline 10 minutes and overlaid with conjugated goat antihuman sera for 20 minutes at 25°C, washed as before, and mounted as described previously. Absorption tests were performed with immune sera by using homologous antigen according to Paterson(9). A Zeiss fluorescent microscope with OSRAM HBO 200 light source was used, with either a BG 12 exciter and GG4-OG4 barrier filters, or UG2 exciter and GG4 barrier filter.

Results. Listeria-immune sera were tested against 30 strains of *Listeria* from both human and animal isolates representing the 4 types of Paterson(9) and type IVb of Seeliger(1). Individual cells fluoresced brightly following treatment with their type-specific labeled sera, but did not fluoresce following exposure to labeled normal sera, *E. insidiosa* antisera, or goat antirabbit. Not all strains tested were detected by the somatic antisera individually. However, a polyvalent serum consisting of equal parts of 7648 (Type I) and 5214 (Type IVa) gave specific fluorescence for all strains. Flagellar antiserum 4428 caused all but one strain to fluoresce, while the other 2 flagellar sera did not produce fluorescence up to 5 strains tested. The whole cell antigen of 4428 was not specific for Types IVa and IVb; however, 7644 provided more specificity in that they gave either a flagellar or a somatic response, or a combination of both. Of all individual sera tested, occasionally a "speckled" appearance was noted, possibly indicating that only a small amount of antigen was present in the cell.

All *Listeria* sera were tested against the following strains by direct technic (figures in parenthesis indicate number of strains tested): *Mimae* sp.(16); *Herellea* sp.(16); *Bacterium anitratum*(10); *Erysipelothrix insidiosa*(28); *Klebsiella pneumoniae*(2); *Salmonella*(8); *Shigella*(6); *Neisseria meningitidis*(16); *N. gonorrhoeae*(6); *Neisseria* sp. (9); *Hemophilus influenzae*(3); *Alkalescens dispar*, Gps. 1-4; *Pseudomonas* sp.(6); *Pasteurella*(8); *Staphylococcus*(21); *Streptococcus*, Gps. A-G; *Corynebacterium*(15). In no instance was there a specific reaction. The bacteria took the counterstain, presenting orange-red characteristics, indicating a non-specific reaction.

Stanley(14), Nyfeldt(15), and others have implicated *Listeria* with infectious mononucleosis. Of 8 sera tested from patients with the clinical disease and a significant heterophile titer, specific fluorescence was obtained from a panel of 16 *Listeria* organisms representing all types. However, to obtain a suitable normal human control serum, we screened 25 normal sera submitted for routine serology and in each instance obtained specific fluorescence, as before. Labeled goat antihuman serum used did not give any specific fluorescence when applied against this panel. Absorbed immune labeled sera did not stain smears of strains of *Listeria* tested. Labeled sera stored at 4°C for 18 months provided a stable reagent.

Comments. The polyvalent somatic serum (7648-5214) provides a suitable medium for detection of all strains tested. The flagellar antigen 7644 can be used to detect a great majority of strains; however, it is recommended that it be used as an ancillary reagent. Whole cell antigens were prepared with the idea not only that they might exhibit the common type III of Paterson(9), but also that we could investigate the findings of Jungherr(16), Webb and Barber(17), and Julianelle and Pons(18), by growing their cultures at 37°C and not at 25°C for flagellar response. Robbins and Griffin(19) state that in 2 sera the common antigen III reaches a high titer in the later part of immunization, but that individual factors cannot be anticipated. Often antibody III completely reverses itself. Paterson(9) indicated that factor III is frequently a "minor factor." Therefore use of a serum of this type is not recommended.

It was not found necessary to precipitate or absorb the sera used, as generally recommended, because the counterstain eliminated all nonspecific fluorescence. Seeliger and Sulzbacher(20) investigated antigenic relationships between serotypes of *Listeria* and *Staphylococcus aureus* by various serologic procedures, finding cross reactions with serotypes I, II, and III. However, in 21 strains tested, no cross reactions were noted.

All serologic procedures have strict limitations in diagnosis of infectious diseases. The fluorescent antibody technic is not without limitations. When one considers the sensitivity based on pneumococcal antigen detectable, as calculated at approximately 10 μg/ml, according to Coons(21), consideration must be given to antibodies present in "so-called" normal serum in amounts detectable by this technic. A "normal" control serum must no longer be considered normal but must also be characterized along with the immune serum. This is also true of antispecies globulins used in the indirect test. As yet there is insufficient information as to minor cross reactions between bacterial, viral, and mycotic agents, heretofore considered unimportant but detectable by this technic. Extreme care must be taken in interpretation of results, using an appropriate confidence-level, adequate controls, and a more complete characterization of sera than is usually necessary.

Agglutination titers can be considered insignificant when applied to this technic. Our investigations indicate that a low titer serum may give a greater degree of specificity than one of higher titer. When using high titer serum, a greater degree of cross reaction is observed. This phenomenon is probably due to minor antigenic factors, enhanced in an attempt to obtain a higher titer. As previously stated(12) bacteria that are not of homologous species take up Lissamine rhodamine nonspecifically. Therefore, when using the counterstain, it is believed that degree of fluorescence cannot be considered of diagnostic significance. Specific fluorescence indicates an antigen-antibody reaction whether it is caused by an antigenic cross reaction or a small amount of the specific antigen, intensity having no particular bearing. Preliminary investigations show that *Listeria* can be detected in clinical material other than pure culture, including formalin fixed tissue, and this may not only prove to be an aid to rapid clinical diagnosis but also provide a means of studying its epidemiology.

Summary. Preparation is described of a polyvalent somatic fluorescein labeled antiserum which was specific for 30 strains of *Listeria* tested but did not react with 180 heterologous strains. Preparation and specificity of flagellar and whole cell antigens are discussed as well as some technical implications of fluorescent antibody technic.

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Propagation of Mouse Polyoma Virus in a Strain of Mouse Mammary Tumor Cells *in vitro*. (25692)

Roslyn Wallace, M. C. Davies, Barbara Pease, Mary Englert and A. W. Moyer
(Introduced by H. R. Cox)
Viral and Rickettsial Research, Biochemistry Research, Lederle Lab., Am. Cyanamid Co.,
Pearl River, N. Y.

The mouse parotid tumor virus(1,2) designated as the S.E. polyoma virus(3), has stimulated much interest because of its oncolytic effect when grown in tissue cultures and its ability to produce multiple tumors in mice and hamsters(4-6). Development of hemagglutinating(7), complement - fixing(8), and mouse antibody production(9) procedures and plaque technics(10-12) provided simple tools for quantitation detection and study of this virus. In the present work tissue cultures of an established cell line of mouse mammary tumor have been used to support growth of polyoma virus, and observations on *in vitro* assay of the virus in these cells are reported.

Materials and methods. Virus. The 1956-8C strain of polyoma virus[*] used here had been cultured through 5 passages in mouse kidney cells. Virus stocks are prepared by inoculating 8 oz. bottles[†] of adult ICR mouse kidney cultures, grown by methods like those of Youngner(13), with 0.5-1.0 ml of undiluted polyoma virus infected tissue culture fluid. The supernatant was saved at each medium renewal and stored at −20°C. When cytopathic changes were nearly complete, cells were collected with the medium and pooled with earlier supernatants. *Tissue culture medium.* "Lactalplus medium," routinely used consists of lactalbumin hydrolysate(14) in modified Earle's salt solution(15), supplemented with vitamins(16), and other components (Table I). To promote cell growth, this medium was further supplemented with 10% calf serum; for maintenance and for viral infectivity titrations, 1% horse serum and $NaHCO_3$ in final concentration of 2.2 g/l were used. All media components were passed through Selas[‡] filters under 5 lb pressure of 5% CO_2 in air. Vitamins and glutamine were prepared at 100 times the desired concentration, frozen at −20°C and incorporated into the medium immediately before use, as were penicillin, 100 μ/ml; streptomycin, 50 μ/ml; and neomycin, 20 μ/ml. *Tissue cultures* were prepared from a strain of cells of mouse mammary tumor (MT) originating spontaneously in a C3H mouse and maintained by serial passage in tissue culture for 7 months.[§] Stock

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[*] Kindly supplied by Dr. S. Stewart and Dr. B. Eddy, Nat. Inst. of Health, Bethesda, Md.

[†] Neutraglas serum bottle #14250, Kimbal Glass Co.

[‡] Microporous porcelain, .02 and .03 porosity. Selas Corp. of America, Phila., Pa.

[§] We are indebted to Dr. Crile Doscher, of Metropolitan Hosp., N.Y.C., and John Turpanjian of these laboratories for establishment and maintenance of this cell line.

TABLE I. Lactalplus Medium.

Ingredient	g/l	Ingredient	mg/l
NaCl	7.0	PABA	.1
KCl	.4	d-Biotin	1.0
$CaCl_2$	.2	Choline	1.0
$MgSO_4\ 7H_2O$	.2	Folic acid	1.0
$NaH_2PO_4H_2O$	.2	Nicotinic acid	1.0
$NaHCO_3$	1.1	Nicotinamide	1.0
Glucose	2.5	Pantothenic acid	1.0
Lactalbumin hydrolysate	5.0	Pyridoxal	1.0
		Riboflavin-5-PO_4	.1
L-cysteine	.05	Thiamine	1.0
Asparagine	.03	Ascorbic acid	50.0
Sodium pyruvate	.10		
L-glutamine	.2		
Phenol red	.001		

cells were grown in 8 oz culture bottles in the growth medium described and subcultured once a week. Experimental cultures were trypsin-dissociated cells from stock cultures resuspended in growth medium. Leighton tubes‖ having a flattened area of 12 x 40 mm near the base, each containing a glass coverslip and 16 x 150 mm tubes were inoculated with 1 ml (approximately 250,000 cells) of cell suspension. Tubes were stoppered and incubated in a nearly horizontal position at 37°C for 48 hours, then renewed with 1 ml of maintenance medium and used. Forty-eight-hour tube cultures of MT cells infected with 0.1 ml of 10^{-1} dilution of stock virus were incubated at 37°C with equal number of uninfected controls. At 2-day intervals the cultures were examined microscopically for cytopathic changes, and 4-8 were selected from both infected and uninfected groups for electron microscopy. From some the fluids were removed, pooled and centrifuged at 500 x g for 15 minutes. From others, the cells were scraped from glass with a 2-5 mm perforated cellophane, and both cells and fluids were collected. Fluids and fluids containing cells were frozen at –20°C for later hemagglutination and tissue culture infectivity titrations. For later study of cytopathic effects (CPE), cover slips were removed from infected and non-infected Leighton tubes at intervals fixed in Bouin's solution and stained with Harris's hematoxylin. Maintenance medium was renewed every 4 days, and the experiment terminated on 12th day. *Assay of virus.* Tissue culture fluids alone were titrated for infectivity in tube cultures of MT cells; fluids containing cells were frozen and thawed 3 times and homogenized in a Ten Broeck grinder before titration. Serial 10-fold dilutions of fluids with and without cells were made in growth medium lacking serum and inoculated in 0.1 ml amounts into each of 2 or 3 cultures. Cultures were held 3 weeks, renewed with maintenance medium each 3 or 4 days, and observed daily for CPE. Titers were calculated by method of Reed and Muench(17) and expressed as number of tissue culture infectious doses ($TCID_{50}$)/0.1 ml. Hemagglutination (HA) tests(18) were performed on heated samples of fluid with and without cells. HA titers were expressed as the highest dilution which caused complete hemagglutination. Hamsters and ICR mice were inoculated within 48 hours of birth with 0.2 ml of original undiluted stock virus and with fluids from cultures infected 8 days. *Electron microscopy.* Cells were fixed by replacing supernatant with 1% osmic acid buffered to pH 7.3 and scraping cells from the glass. After a 50% alcohol rinse, they were dehydrated by successive applications of 70, 95 and 100% alcohol, and small portions of sediment were embedded in 75% butyl 25% methyl methacrylate. Sections cut on a Porter-Blum microtome with glass knives and picked up on carbon-filmed specimen grids, were observed with an RCA EMU3C microscope.

Results. Light microscope observations. CP effects in infected MT cultures during 12 days incubation were noted in Table II. Uninfected cells varied from polygonal to spindle-shaped and, where less crowded, showed connecting protoplasmic processes. Nuclei were large and prominent; giant cells and multinucleated cells were sometimes seen (Fig. 1). CPE seen earliest 6 days after infection was characterized by scattered necrotic cells, somewhat rounded, shrunken, and with densely staining nuclei. Cytoplasm around many of these nuclei appeared fragmented, and no intact cell wall could be seen. Subsequent ratings of CPE were based primarily on increasing number of necrotic cells and cellular debris. Ten days after infection, fragments of densely staining cells and of destroyed cells peppered the culture. Some ap-

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‖ Microbiological Assoc., Bethesda, Md.

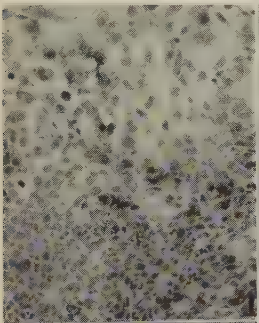
TABLE II. Correlation of Cytopathic Effects with Tissue Culture Infectivity and Hemagglutination Titers of Polyoma Virus on Mouse Mammary Tumor Cells *In Vitro.* (Original inoculum 4.5 $TCID_{50}$)

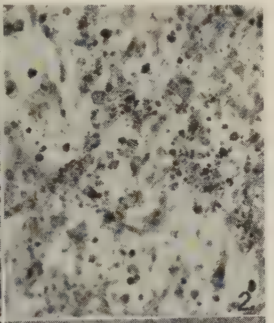
Days incubation	CPE	Tissue culture infectivity titers*		Hemagglutination titers†	
		Fluid only	Fluid + cells	Fluid only	Fluid + cells
2	0	4.0	4.0	0	0
4‡	0	4.5	4.5	0	0
6	<+	4.5	5.0	1:16	1:256
8‡	2+	5.0	6.0	1:256	1:1024
10	2-3+	5.5	6.5	1:256	1:2048
12	4+	5.5	6.5	1:512	1:2048

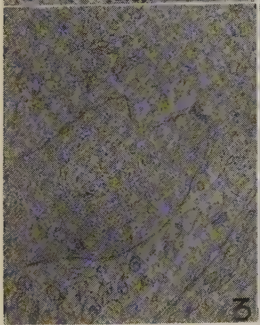
* Tissue culture infectivity titers expressed as log 10 of $TCID_{50}/0.1$ ml.
† Hemagglutination titers expressed as highest dilution causing complete hemagglutination.
‡ Culture fluid renewal.

parently unaffected cells could also be seen (Fig. 2). After 12 days of infection, most cells appeared necrotic and disintegrating.

Virus HA and tissue culture titrations. Infectivity titrations in tissue culture and results of HA tests on tissue culture fluids from infected cultures, Table II, clearly show that polyoma virus multiplied and that highest levels of virus production and release began around 8 days after infection. Titrations of centrifuged fluids and fluids containing disrupted cells show also that a significant amount of the virus produced remains associated with cells and is not released immediately into the medium. This observation supports that made by Sachs on polyoma-virus-infected mouse embryo cells(19). Virus production appears to accompany cytopathic changes observed under light microscope. Failure of fluids from 2-day and 4-day cultures to give positive HA results is not clearly understood. Positive HA results were obtained with tissue culture fluids beginning 6 days post-infection, *i.e.,* with early cytopathology and probably early virus production. Tissue culture titrations, however, revealed presence of virus (possibly entirely that introduced with original inoculum) in both 2-day and 4-day culture fluids.







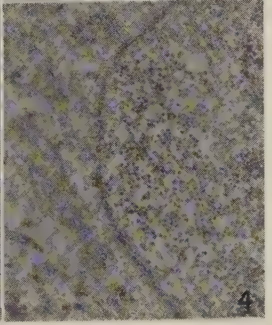


FIG. 1. Uninfected mouse mammary tumor cells incubated 12 days. Harris' hematoxylin stain. × 87.

FIG. 2. Mouse mammary tumor cells 10 days after infection with polyoma virus. Harris' hematoxylin stain. × 87.

FIG. 3. Portion of uninfected mouse mammary tumor cell showing large irregular nucleus with marginated chromatin and small mitochondria. × 5280.

FIG. 4. Portion of cell 12 days after infection showing concentration of virus particles within nucleus and necrotic state of cytoplasm. × 12,820.

Electron microscope observations. In uninfected cells, relatively large irregular nuclei could be seen. Chromatin was unevenly distributed, tending to concentrate near the nuclear membrane. The cytoplasm contained many small mitochondria generally ellipsoid in shape (Fig. 3). Six days after infection the cytoplasmic components of some cells began to show loss of structure and, in some thin sections, virus-like particles could be seen in the enlarged nucleus. Eight days after infection the majority of cells were infected; particles were numerous in the nuclei, and occasionally a few were seen in the cytoplasm. Whether these latter emerged from the nucleus is not known. The intranuclear particles appeared to increase rapidly with a concomitant necrosis of the cytoplasm. About 12 days after infection in those cells which had not been lysed, virus particles practically filled the nucleus, but showed no tendency to form geometric arrays (Fig. 4). They averaged approximately 30 mμ in diameter, and no internal structure has been seen.

Animal inoculations. On the whole, tumor production in hamsters and mice with this strain of virus has been disappointing. Out of 4 hamster litters (20 animals) inoculated with original stock virus, only 7 animals survived weaning of which 2 developed a solid subcutaneous tumor mass at site of inoculation at 2 months of age, and one developed multiple neoplasms and heavy ascitic fluid at 3 months of age. Out of 4 litters (18 hamsters) inoculated with tissue culture fluid collected from cultures 8 days after virus infection, 9 animals survived weaning. Two of these developed solid tumor masses at site of inoculation at 2.5 months of age, and one developed tumor of heart and liver at 3.5 months. Out of 8 ICR mouse litters (48 animals) inoculated with original undiluted stock virus and tissue culture fluids collected 8 days after virus infection, 31 animals survived weaning. Of these, 3 developed multiple tumors (parotid and mammary gland, heart and liver) at 4 months, and 2 developed parotid gland tumors only at 4.5 months. No animals receiving uninfected tissue culture fluids have developed tumors after 4.5 months.

Discussion. This strain of MT cells is particularly useful for tissue culture titrations of materials infected with polyoma virus. Infected cultures can be maintained in a satisfactory condition for 3 weeks or more in medium containing low concentrations of serum —a factor of some importance because of inhibiting properties of serum on this virus(11). Usefulness of a milk-adapted strain of P388 mouse lymphoma cells for studies on polyoma virus has been noted(20). Primary cultures of whole mouse embryo or adult mouse kidney generally used for this purpose may be less desirable because of the possible introduction of latent mouse viruses. Uninfected control cultures of the MT cell line have not shown cytopathic effects due to polyoma virus infection as sometimes observed in control tubes of primary whole mouse-embryo cultures. Moreover, separately prepared batches of tube cultures from stock MT cell strain gave satisfactorily uniform results on titrations of virus activity in contrast to irregular results sometimes observed with primary mouse-embryo cultures(9).

That polyoma virus CPE on tissue culture cells is accompanied by virus production agrees with the report of Eddy *et al.*,(3) who demonstrated that only fluids from infected tissue cultures which showed cytopathic changes produced tumors in hamsters. The relatively low incidence of tumors induced by these preparations is presumably due to the considerable number of serial passages of virus in tissue culture and accords with the findings of Drs. W. P. Rowe and S. E. Stewart.¶ Neutralization tests in tissue cultures of MT cells with sera from several mice and hamsters selected from our stock animals did not indicate presence of polyoma antibodies. Three different tissue culture passages of this strain of polyoma virus, one representing 7 passages in MT cells, one representing 4 passages in mouse kidney cultures, and one representing 5 passages in whole mouse-embryo cultures were completely neutralized in hemagglutination-inhibition assay(21) by a sample of polyoma virus antiserum kindly supplied by Dr. W. P. Rowe.

Summary. 1) An established cell line originating from a spontaneous mouse mammary tumor is a convenient host in which to propagate and to assay polyoma virus *in vitro*. Cytopathic effects of the virus were first observed in cultures 6 days after infection and progressed to complete destruction of all cells by 12th day. Increasing cell destruction was accompanied by rise in tissue culture infectivity and hemagglutination titers. 2) Observations with electron microscope revealed presence of virus in infected cells beginning 6th day after infection. Virus particles were observed in the nucleus and occasionally in the cytoplasm. Particles averaged 30 mμ in diameter and did not show an internal structure.

¶ Personal communication.

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Electrolytes in Denervated and Reinnervated Muscle. (25693)

Z. Drahota (Introduced by A. M. Shanes)
Inst. of Physiology, Czechoslovak Academy of Sciences, Prague

Since the work of Hines and Knowlton(1) on ion changes in the denervated muscle it has not been clear whether the decrease of K ions in the denervated muscle is the result of a decrease in intracellular K concentration(2) or is only proportional to diminution of the muscle phase itself and to increase in relative amount of intercellular collagen phase(3). The content of extracellular ions Na and Cl increases after denervation when expressed in mequiv. Na and Cl/100 g fat-free dry solid (FFDS)(1,2,3). However, ion changes in denervated muscles have not been evaluated in absolute units, *i.e.*, total amounts of Na and Cl ions present in whole normal and denervated muscles have not been compared. Such an evaluation makes it possible to study the relationship between extra- and intracellular muscle phases in denervation atrophy. Moreover, if ion composition of the denervated muscle is changed, it is very important to know whether and how it returns to normal in the course of reinnervation. These aspects are the subject of this report.

Methods. Experiments were performed on male rats of 150-200 g body weight. Ion composition was studied in extensor digitorum longus following section of peroneal nerve. For reinnervation studies, denervation was induced by crushing the sciatic nerve; this procedure leads to reinnervation of the muscle and restitution of function about 14 days after nerve crushing(4). For these experiments the anterior muscle was used. Na and K ions were determined by flame photometry, Cl ions by modification of Sanderson's method(5). Non-collagenous protein nitrogen was extracted from the muscle with 0.1 N NaOH, precipitated with TCA, and after Kjeldahlisation was determined by Conway's micromethod(6).

Results. Decrease in non-collagenous protein nitrogen occurs in denervated muscle at the same rate as the decrease in K content

TABLE I. Potassium and Non-collagenous Protein N Content in Denervated Muscle.

Denervation period, days	Control muscle	Denervated muscle	Denervated / Control
	μequiv. K/muscle		
3	8.63 ±.76	8.89 ±.80	1.03
20	11.55 ±.06	7.32 ±.06	.63
	mg N/muscle		
3	2.39 ±.18	2.34 ±.13	.98
20	3.52 ±.88	2.20 ±.07	.63
	K/N		
3	3.61 ±.13	3.80 ±.19	1.05
20	3.28 ±.19	3.32 ±.13	1.01

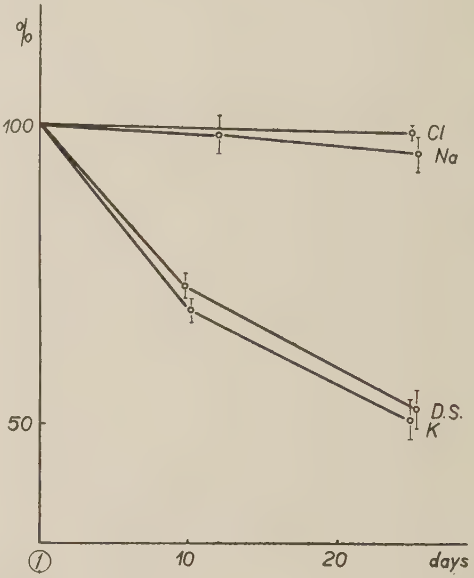
All data are mean values of 6 muscles.

(Table I). The amount of K corresponding to 1 mg protein nitrogen remains unchanged after denervation. Since non-collagenous protein nitrogen corresponds to the intracellular muscle mass, our results thus confirm earlier assumptions of some authors who, finding decreased K content in the denervated muscle, concluded that this decrease is caused by a relative increase of collagenous tissue and a decrease in the muscle phase/unit weight of denervated muscle(1,3).

On evaluating Na and Cl ions in absolute units, *i.e.*, per whole muscle, one finds that total amount of Na and Cl ions remains unchanged in the denervated muscle (Fig. 1). Changes in K correspond well with results in Table I. Rate of K decrease and rate with which fat-free dry muscle solids decrease run almost parallel.

Ion composition of the denervated muscle gradually returns to normal with reinnervation (Fig. 2). When evaluated in absolute units (total amount/muscle), sodium ions remain unchanged throughout reinnervation period. The K content (μequiv./muscle) and FFDS (mg/muscle) diminish concomitantly during denervation; after restitution of muscle motor function they recover together.

Discussion. Intracellular water (ml/100 g FFDS) in the denervated muscle remains unchanged, as does intracellular sodium content (Na/100 gr. FFDS), hence the increase in Na ions, when expressed in relation to total dry mass, corresponds to the change in relative amount of extracellular fluid(7,8). Taken together with our data on the main intracellular ion, K, as given in Table I, available results show that osmotic conditions remain unchanged in muscle cells deprived of their motor innervation. Twenty-five days after denervation muscle atrophy reaches 50% (Fig. 1). At this time marked metabolic disturbances take place inside muscle cells(9). In spite of this, however, the transport sys-



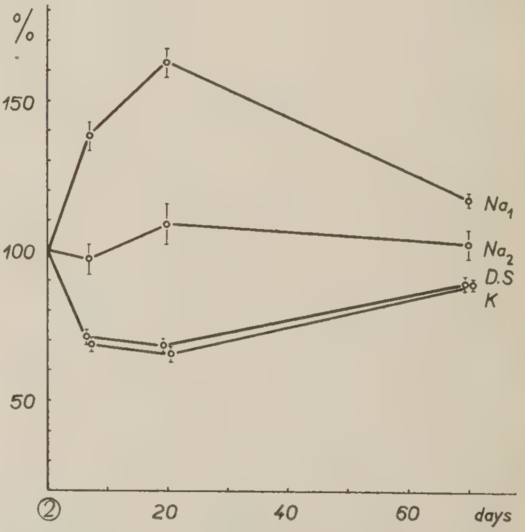


FIG. 1. Changes in electrolytes (Cl, Na, K) and fat-free dry solids (D.S.) after denervation. Na, Cl, K, D.S. represent % changes of these relative to normal innervated control muscles.

FIG. 2. Na, K, and fat-free dry solids (D.S.) changes during denervation followed by reinnervation. Na_1 represents % change of Na expressed in meq/100 g FFDS. Na_2, K, D.S. represent % changes of total amounts/muscle.

tems of cells maintain concentration gradients of Na and K ions under these conditions at normal level even though rate of K exchange in the denervated muscle is somewhat decreased(10).

Evaluation of total absolute ion changes makes it possible to estimate changes in extracellular fluid and the intracellular muscle phase. The results indicate that following denervation, only the intracellular muscle phase decreases, while extracellular space remains unchanged even when muscle atrophy reaches 50%. Conclusions in earlier reports concerning increase of Na and Cl ions in the denervated muscle(1,2,3,7,8) must be modified in view of our evidence that the increase of Na and Cl ions/unit weight of denervated muscle is only relative, due to volume of extracellular fluid remaining unchanged while volume of intracellular phase decreases.

Summary. The content of potassium expressed in relation to non-collagenous protein nitrogen is normal even 20 days after denervation in muscles of the rat. Following denervation only the intracellular muscle phase decreases. The drop in K content (K/muscle) and FFDS (weight/muscle) reaches 50%. At this time the extracellular space (Na/muscle, Cl/muscle) remains unchanged. Ion composition and dry muscle weight gradually return to normal after reinnervation and restitution of motor function.

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Histological Changes in Cells Infected with Columbia SK Virus. (25694)

EIICHI FURUSAWA, AND WINDSOR CUTTING
Dept. of Medical Microbiology, Stanford University

Histological evidences of Columbia SK virus infection have been of interest because the nature of pathological changes and site of multiplication of the virus have not been completely identified, and also because we hoped to identify the step at which virus propagation was interrupted by the antiviral substance "Propionin"(1). Some clarification of the first of these problems is given in this paper, in which histological appearance of infected cancer cells is described.

Procedure. After serial passage in Ehrlich ascites tumor cells, grown intraperitoneally in young white mice, an adapted strain of Columbia SK virus was obtained which produced severe oncolysis(2). Such infected ascites fluid, aspirated with 2 ml of saline from abdominal cavity before mice died at 3 to 5 days after virus inoculation, contained about 10^6 LD_{50}/ml of virus. The supernatant of this fluid, which contained the same titer of virus, was diluted 10^{-3}, and 0.1 ml mixed with 0.3 ml of fresh, uninfected Ehrlich ascites, drawn 5 to 10 days after implantation, and inoculated intraperitoneally into new mice. Three or 4 days later cells were withdrawn for morphological study, and stained as follows: *Giemsa*—Smears were fixed with methanol and stained by usual Giemsa solution. *Lipoid*—Smears were fixed with formalin and stained with Sudan III, or Sudan Black B, saturated solutions in 70% alcohol, then counterstained with Giemsa solution. *Tetrazolium*—Immediately after aspiration a drop of ascitic fluid was mixed on a slide with a drop of 2% 2,3,5-triphenyl-2H-tetrazolium chloride solution in saline. A cover slip was applied, ringed with petrolatum, and the slide examined after 30 minutes at room temperature. *Acridine orange*—Smears were fixed with formalin and stained with 0.1% acridine orange R at pH 3.0, adjusted with HCl by the method of Armstrong(3), for 30 minutes, then washed with water and dried. *Fluorescent antibody*. The indirect method of Weller and Coons(4) was applied, using immune rabbit serum against Columbia SK as previously described(2), including 2 adsorptions of nonspecific substances by washed Ehrlich ascites cells, and producing an anti-hemagglutinin titer of 1:640. Fluorescein conjugated sheep anti-rabbit globulin was obtained commercially. Smears were fixed with acetone for 10 minutes, then exposed 30 minutes to 1:10 dilution of anti-SK rabbit serum diluted with phosphate buffered saline. After washing gently with buffered saline, they were overlaid for 30 minutes with a 1:10 dilution of fluorescein-conjugated sheep globulin. After final washing, smears were mounted in buffered glycerin and examined for presence of intracellular antigen under fluorescence microscope with a dark field condenser. Normal rabbit serum replaced anti-SK rabbit serum in controls.

Results. Characteristic appearances of normal and infected tumor cells are shown in Fig. 1. Progress of the infection as stained by Giemsa solution is shown in 1, 2 and 3. No. 1 shows uninfected or control tumor cells; No. 2, 2 days after infection, shows extensive vacuolization and loculation of the cytoplasm, in which many vacuoles are larger than those of control cells; No. 3, 4 days after infection, indicates massive oncolysis present in late stages of infection, disintegration of cytoplasm, and shrinking and distortion of the nuclei. Nos. 4, 5 and 6 show the same stages stained by Sudan Black B. A number of stained vacuoles are seen in control cells of No. 4 but these appear not to be the same vacuoles as the smaller type seen by Giemsa staining; No. 5, 2 days after infection shows a moderate increase in Sudan Black B stained vacuoles, indicating an increase in lipoid; terminal stages, in No. 6, show the still greater accumulation present in some cells, but virtual absence in others, highly loculated, which may indicate loss of fat before disintegration. No. 9, by contrast, stained with Sudan III, in a cell 4 days after infection, shows presence of neutral fat in the large terminal locules. The difference in staining with the 2 fat stains is definite but not explained. Normal cells show little or no staining with Sudan III. Nos. 7 and 8 show normal tumor cells stained with triphenyltetrazolium chloride, and infected cells at 2 days; the brilliant red color produced by reduction of the dye in vacuoles of controls is entirely absent in infected cells. No. 10 shows uninfected cells stained with acridine orange, as seen with fluorescent microscope; nuclei are green and cytoplasm brilliant orange. After 2 days, in No. 11, the usual intense vacuolization is seen, vacuoles being unstained, and some cells have lost most of their orange coloration; after 4 days, in No. 12, green ghosts may be seen and only a single normal, or recently infected, cell with orange cytoplasm. Finally, Nos. 13, 14 and 15 show increase of fluorescent antibody as the disease progresses 2, 3, and 4 days after infection. Initial fluorescence is peripheral, in small areas, but this evidence of virus antigen progresses steadily until nearly the whole cell may be involved.

These results indicate that infection produces: a. vacuolization and loculization (Giemsa), b. increase of lipoid vacuoles (Sudan Black B and Sudan III), c. failure of reduction of tetrazolium (tetrazolium), d. disappearance of cytoplasmic ribonucleic acid (acridine orange), and e. gradual increase in virus antigen, probably starting in the cytoplasm (fluorescent antibody).

Discussion. Two processes go on simultaneously in the infected cells described, *i.e.*, destruction of the cell and propagation of the virus. Positive evidence of virus propagation was afforded only by the fluorescent antibody technic as other methods of staining showed what more probably were manifestations of damage to the cells. If the Columbia SK virus is an RNA virus, like the polio virus which it resembles in some respects, the disappearance of ribonucleic acid stainable by acridine orange is unexpected and might be interpreted as the result of replacement of normal RNA

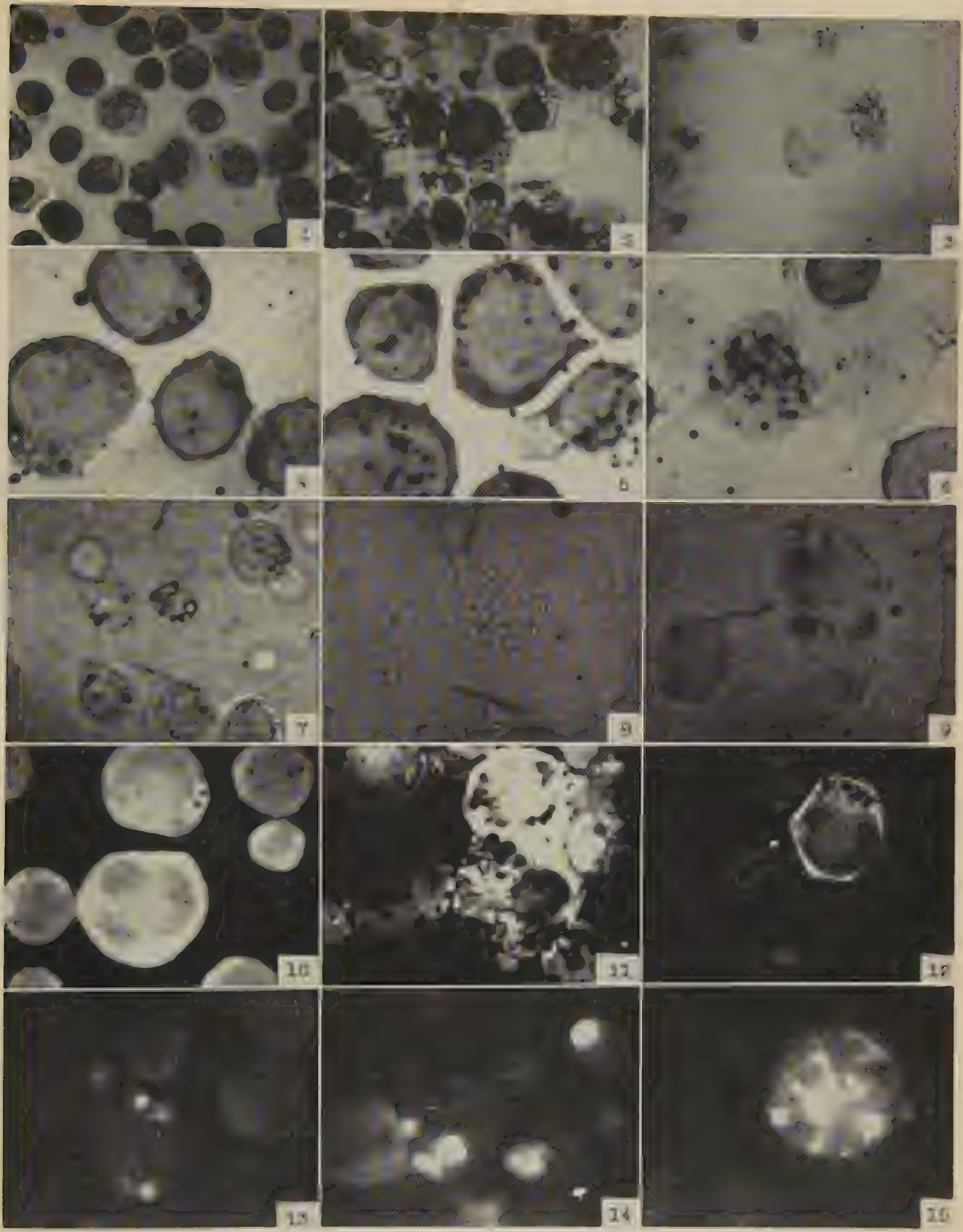

FIG. 1. Ehrlich ascites cells showing changes produced by Columbia SK virus infection (see text for details).

of cytoplasm by virus in which RNA was occluded by a protein matrix or the protein viral coat. However, Niven(5) has shown that acridine orange can penetrate through protein to stain virus RNA in some cases. The changes of lipoid and tetrazolium staining indicate enzymatic aberrations, probably part of the oncolytic process, which might be subject to further analysis by enzymatic and metabolic studies. It is hoped that the histological changes described will clarify the point of action of chemotherapeutic agents.

Conclusions. The changes produced in Ehrlich ascites tumor cells by Columbia SK virus are described, using various staining technics including fluorescent antibody staining.

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Protamine and Conversion of Mevalonic Acid to Cholesterol by Liver Homogenates.* (25695)

LEMUEL D. WRIGHT AND ROSEMARIE HANSON
Graduate School of Nutrition, Cornell University, Ithaca, N. Y.

Preincubation of essentially whole homogenates of rat liver with crystalline ribonuclease (RNAse) abolishes capacity of these homogenates to convert mevalonic acid (MVA) into non-saponifiable material (NSF) or cholesterol (CHL)(1). Evidence has been presented that RNAse acting in association with easily sedimentable constituent of homogenate (2,3) destroys a system concerned with maintenance of ATP(4,5,6). This system appears to require a polynucleotide, presumably "free" or "combined" RNA as an essential component since inactivation may be prevented or reversed by a "nucleoprotein" of autoclaved liver extract, or by relatively large amounts of RNA, or by DNA, or by a number of "unnatural" anionic polymers including polyethylene sulfonate (PES) or heparin(7,8). It has now been found that capacity of the system for maintaining adequate ATP levels as judged indirectly by utilization of MVA for biosynthesis of NSF is lost by preincubation of the homogenate with protamine. This protamine inhibition is associated also with absence of ATP in the homogenates and is, furthermore, preventable with RNA, DNA, or PES and, using PES as an example, is associated with a level of ATP in the homogenate equal to that of control. Reasons are presented here for believing that preincubation of liver homogenates with protamine results in formation of a protamine-RNA complex that renders RNA unavailable for its role in maintenance of ATP levels adequate for phosphorylation of MVA.

Methods and materials. The biosynthetic experiments involved preincubation of 5 ml aliquots of a 200 x g supernatant fraction of rat liver homogenate prepared as previously described(1-9) with 5-8 ml amounts of solution containing 1 mg ATP, 10 mg DPN, 100 mg sodium succinate, various amounts of protamine (neutralized), and other supplements to be studied. Each flask was aerated with a stream of oxygen and preincubated with agitation for 30 min at 37°. Following preincubation each flask was opened and 1 ml MVA-2-C^{14} solution added, the contents were aerated and the flasks then reincubated for additional 3 hrs. After final incubation, homogenates were saponified, extracted with petroleum ether, the ether extracts dried with sodium sulfate, filtered, evaporated, taken up in scintillation mixture and counted. Previous studies have shown(1,9) that under experimental conditions employed the counts found in the NSF fraction are essentially CHL or other digitonin-precipitable material. In experiments involving determination of ATP

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TABLE I. Biosynthesis of NSF by Liver Homogenates as Influenced by Preincubation with Protamine or Protamine and Various Anionic Polymers.

Exp. No.	Protamine, mg	Additional supplement, mg	NSF, cpm
1	0	None	8254
	1	"	8148
	2	"	8011
	5	"	40
2	0	"	5982
	1	"	5862
	2	"	5889
	5	"	161
	10	"	75
3	0	"	7652
	5	"	6709
	10	"	89
	0	PES, 5 mg	6474
	5	*Idem*	6693
	10	"	7089
	0	DNA, 5 mg	7480
	5	*Idem*	7863
	10	"	162
	0	RNA, 5 mg	8078
	5	*Idem*	9202
	10	"	97
4	0	None	7962
	10	"	489
	"	PES, 5 mg	7500
	"	10	7384
	"	20	7394
	"	DNA, 5	1337
	"	10	8597
	"	20	8795
	"	RNA, 5	521
	"	10	855
	"	20	7154
5*	0	None	9332
	10	"	64
	10	PES, 5 mg	8545

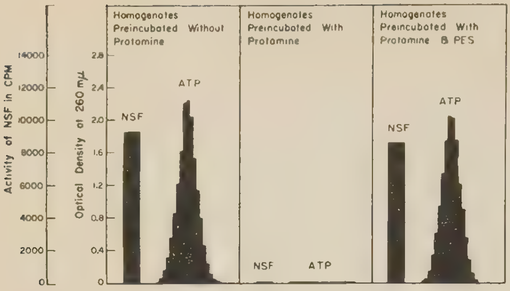
* In this experiment supplementation of homogenate with additional ATP was intentionally omitted.

content of homogenates at time of MVA addition following preincubation, the contents of paired flasks were treated with equal volume of cold 4% perchloric acid. The precipitate that formed was removed by centrifugation and the supernatant solutions were freed of excess perchloric acid by careful neutralization with cold 10% potassium hydroxide. The solutions were centrifuged to remove potassium perchlorate and supernatant solutions were subjected to anion exchange chromatography and gradient elution on Dowex-1 as described by Allfrey and Mirsky(10) and used previously(4). ATP was determined spectrophotometrically in the eluates. In biosynthetic experiments involving effect of added ATP the ATP was added to homogenates following preincubation at time of MVA addition(5). The influence of protamine on activity of ATP *in vitro* was determined by coupling hexokinase and glucose-6-phosphate dehydrogenase reactions under conditions where an increase in optical density at 340 mμ from TPNH is a measure of ATP availability(11). Protamine sulfate used was a commercial preparation (Mann Research Labs). The polyethylene sulfonate (Upjohn) had a mean molecular weight of 12,900. ATP was used as crystalline disodium salt (Pabst Labs).

Results. Biosynthesis of NSF from MVA is completely inhibited by preincubation with about 5 mg of protamine/flask (Table I). Inhibition produced by as much as 10 mg of protamine is preventable with 5 mg of PES. DNA and RNA are less active. In one experiment 5 mg of DNA showed detectable activity and 10 or 20 mg showed complete activity against 10 mg of protamine. In the same experiment 10 mg of RNA showed detectable activity and 20 mg showed complete activity against this level of protamine.

Fig. 1 shows that a control homogenate of liver preincubated without protamine had an adequate level of ATP (calculated to be 2 mg) as determined by ion exchange chromatography while a paired flask supplemented with MVA following preincubation was highly active in conversion of MVA to NSF. On the other hand, a homogenate preincubated with protamine was devoid of ATP and a paired flask supplemented with MVA following preincubation was inactive in conversion of MVA to NSF. Homogenate preincubated with protamine plus PES contained a level of ATP equal to the preincubated control homogenate (2 mg) and conversion of MVA to NSF in a paired flask was equal to that in control.

Additional evidence that preincubation of a homogenate with protamine results in a system that does not maintain a level of ATP is presented in Table II. In this experiment paired flasks were preincubated with 0, 5 and 10 mg of protamine. Following preincubation 10 mg of ATP was added to one flask of each pair at time of MVA addition. As noted in



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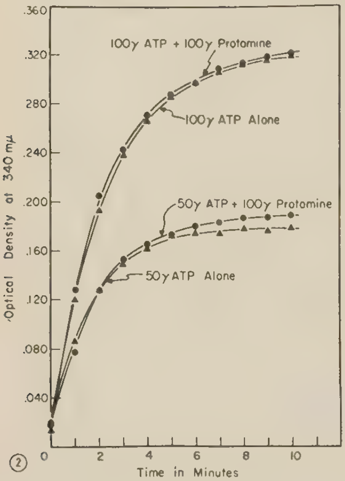

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FIG. 1. ATP content of rat liver homogenates preincubated without protamine, preincubated with protamine, or preincubated with protamine and PES. Activity of NSF isolated from paired flasks to which MVA-2-C^{14} was added following preincubation also is indicated.

FIG. 2. Absence of an effect of protamine on availability of ATP for phosphorylation of glucose to glucose-6-phosphate by hexokinase as determined by TPNH formation in coupled oxidation of glucose-6-phosphate to 6-phosphogluconolactone by zwischenferment.

earlier experiments, preincubation of homogenates with protamine is associated with an almost complete absence of MVA incorporation into NSF (8651 cpm in control *vs.* 39 and 49 cpm with 5 and 10 mg protamine). In agreement with previous studies involving ATP(5), addition of ATP in this way to control homogenates is associated with some reduction in capacity to convert MVA to NSF. (5932 cpm with ATP *vs.* 8651 cpm without ATP). However, conversion of MVA to NSF in the flask containing 5 mg of protamine to which ATP was added was essentially equal to that in the control homogenate with ATP (4921 cpm *vs.* 5932 cpm or 4921 cpm with ATP *vs.* 39 cpm without ATP) while in the flask containing 10 mg of protamine subsequently supplemented with ATP there was marked conversion of MVA to NSF (1931 cpm with ATP *vs.* 49 cpm without ATP). The active conversion of MVA to NSF in flasks preincubated with protamine and then subsequently supplemented with ATP as compared with the unsupplemented flasks is evidence that the first inhibitory effect of protamine in homogenates is against a system concerned with maintenance of an ATP level.

Since the inhibitory effect of protamine is preventable with RNA, DNA or PES and is reversible with ATP the possibility existed that protamine functions by combining with either or both types of compounds *i. e.,* the relatively large anionic polymers or the relatively small coenzyme. Evidence that protamine has no effect on at least one biochemical function of ATP is furnished in Fig. 2. In this experiment ATP was made the limiting factor in phosphorylation of glucose by hexokinase and this reaction was coupled to oxidation of glucose-6-phosphate by zwischenferment where TPN reduction as measured by absorption at 340 mμ could be used to measure rate and extent of phosphorylation. At levels employed (protamine: ATP ratio of 1 or 2) protamine is without effect on rate or extent of ATP activity. These results indicate that the inhibitory effect of protamine in the biosynthetic experiments is against compounds concerned in maintaining a level of ATP rather than against ATP itself.

Discussion. Our data show that preincubation of liver homogenates with protamine yields a system that does not convert MVA to NSF (largely CHL). Inactivation may be prevented by carrying out preincubation with

TABLE II. Influence of ATP on Biosynthesis of NSF from MVA in Homogenates Preincubated with Various Levels of Protamine.

	Activity of NSF, cpm	
Protamine, mg	Unsupplemented homogenates	ATP supplemented homogenates*
0	8651	5932
5	39	4921
10	49	1931

* 10 mg ATP added/flask at time of MVA addition following preincubation with indicated level of protamine.

protamine in the presence of added RNA, DNA or PES. Control homogenate preincubated without protamine maintained a level of ATP while homogenate preincubated with protamine was devoid of ATP. Homogenate preincubated with protamine plus PES maintained a level of ATP equal to the control. Protamine inhibition also could be reversed with large amounts of ATP. Evidence that protamine combines with factors, presumably "free" or "combined" RNA, concerned with maintenance of ATP levels rather than with ATP *per se,* was found by activity of ATP in an *in vitro* enzyme system that requires ATP was not influenced by protamine to ATP levels comparable to those involved in the biosynthetic experiments. Our findings lead to the interesting question, implied or discussed by a number of authors, of the extent to which control of biochemical activity within the cell is regulated *via* basic proteins. It appears that if a strongly basic protein such as protamine were elaborated intracellularly activity requiring ATP might be depressed as a consequence of unavailability of "free" or "combined" RNA. On the other hand, some degree of RNA-protein combination appears essential to protect RNA from intracellular RNAse. Our studies have shown that "nucleoprotein" of autoclaved liver extract is resistant to RNAse but after separation of the moieties the RNA is then susceptible to RNAse. Thus a number of factors that have been loosely characterized in the literature dealing with "tightness" with which phosphorylation is coupled (or uncoupled) to oxidation may represent proteins of varying affinity for polyanions of the cell that seem to be concerned with maintenance of ATP levels.

Summary. Protamine is a potent inhibitor of a system essential for conversion of mevalonic acid to cholesterol. Evidence was presented that protamine combines with a polyanion, presumably "free" or "combined" RNA, that is essential for maintenance of an ATP level required in utilization of mevalonic acid.

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Effect of Neomycin on Cholesterol Atherosclerosis in the Rabbit. (25696)

EDWIN R. FISHER*
Depts. of Pathology, University of Pittsburgh and Vet. Admin. Hospital, Pittsburgh, Pa.

A significant decrease in serum cholesterol has been recently observed in 10 patients following oral administration of the antibiotic Mycifradin which contains 70% neomycin sulfate(1). However, Broitman *et al.*(2) observed a marked hypercholesterolemia and valvular sudanophilia in neomycin-treated rats fed a cholesterol and cholate diet. In both studies the effect of neomycin was attributed to alterations of intestinal bacterial flora or certain enzyme systems of the gastrointestinal tract. The purpose of this report is to note the effect of oral and parenteral administration of this agent on serum lipids of normal and cholesterol-fed rabbits and the

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* Senior Research Fellow U.S.P.H.S.

TABLE I. Effect of Neomycin on Body Weight, Coliform Count of Stool and Aortic Atherosclerosis.

Group		Diff. wt (kg)	Coliform count/g		Degree of As*
			Beginning	End	
I	Oral neomycin + chol.	+ .6	6.2×10^8	4.1×10^2†	1.75
II	Chol.	+ .5	4.3×10^7	6.6×10^8	.75
III	Intramusc. neomycin + chol.	+ .7	5.2×10^7	3.6×10^7	.83
IV	Oral neomycin	+1.0	7.3×10^8	6.0×10^3†	0
V	Intramusc. neomycin	+1.1	6.2×10^7	4.7×10^6	0

* Avg of thoracic and abdominal segments (maximum 3.0). † $P < .01$.

atherosclerotic process associated with the latter.

Material and method. Forty adult, white rabbits initially weighing 2 kg were divided into 5 groups, each containing equal numbers of both sexes. Group I consisted of 12 animals maintained on 2% cholesterol diet and drinking water containing 0.5% (w/v) neomycin[†] Eight rabbits of Group II maintained on cholesterol diet and drinking water without neomycin. Group III comprised 8 animals receiving cholesterol diet and daily intramuscular injections of 50 mg of neomycin. Group IV consisted of 6 animals maintained on stock laboratory diet without cholesterol supplement and drinking water containing 0.5% neomycin. Group V consisted of 6 animals maintained on stock cholesterol-free diet and tap water but received daily intramuscular injections of 50 mg of neomycin. All animals were sacrificed after 70-72 days of treatment. Animals receiving neomycin orally consumed a total of 30-40 g of the drug during experimental period. Blood was obtained by cardiac puncture prior to sacrifice and total and esterified cholesterol determined by Schoenheimer and Sperry method(3), total lipids according to de la Huerga *et al.*(4), and lipid phosphorus by the method of Youngburg and Youngburg(5). Coliform counts were made on samples of stool before initiation of experiments and at time of sacrifice. Degree of aortic atherosclerosis was estimated at necropsy as described previously(6). Organs and aorta were routinely stained with hematoxylin and eosin after fixation of tissue in 10% neutral formalin. Blood pressure was determined on ½ of animals of each group by the ear-capsule technic of Grant and Rothschild(7).

Results. No toxic effects of neomycin were noted. All animals receiving cholesterol diet exhibited less weight gain than rabbits maintained on cholesterol-free, stock laboratory diet although food consumption was considered comparable (Table I). Prolonged administration of neomycin orally or parenterally failed to influence these changes in body weight. Coliform counts on aliquots of stool were significantly reduced only in animals receiving neomycin orally whether maintained on cholesterol or stock laboratory diets. Elevation of serum lipids (Table II) attendant with cholesterol feeding was significantly greater in rabbits subjected to oral neomycin (Group I) than in those of Group II that did not receive this drug. Similarly, aortic atherosclerosis as indicated in Table I and Fig. 1 and visceral lipoidosis were greater in the former (Group I). Serum lipids and degree of atherosclerosis were similar in cholesterol-fed rabbits subjected to intramuscular injections of neomycin (Group III) and those not receiving this drug (Group II). Administration of neomycin to animals not receiving cholesterol failed to alter their serum lipids or normal appearance of the aorta and viscera. All animals remained normotensive and differences observed were not related to sex.

Histologic study failed to disclose significant qualitative differences in atherosclerotic lesions encountered in neomycin-treated or untreated animals fed cholesterol. No evidence of renal damage was apparent in animals receiving neomycin.

Comment. The effect of orally administered neomycin in cholesterol-fed rabbits appears similar to that observed in the rat by Broitman *et al.*(2), producing a greater eleva-

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[†] Administered as Mycifradin Sulfate; generously furnished by Upjohn Co.

TABLE II. Effect of Neomycin on Serum Lipids.

Group	No.	Cholesterol (mg %) Total	Cholesterol (mg %) Esters	Total lipid (mg %)	Lipid P (mg %)
I Oral neomycin + chol.	12	1580 ± 229	666 ± 143	2275 ± 250	32 ± 6
II Chol.	8	938 ± 264	373 ± 140	1573 ± 254	20 ± 8
III Intramusc. neomycin + chol.	8	963 ± 218	390 ± 80	1370 ± 230	19 ± 7
IV Oral neomycin	6	110 ± 10	68 ± 10	247 ± 63	3 ± .05
V Intramusc. neomycin	6	101 ± 6	61 ± 6	210 ± 71	3 ± .06

P groups I & II, I & III <.01. P groups II & III, IV & V >.1.

tion of serum lipids and a more severe degree of aortic atherosclerosis than in untreated, cholesterol-fed controls. Accentuation of visceral lipoidosis was also observed in orally treated, cholesterol-fed rabbits. Although oral administration of neomycin resulted in marked elevation of serum lipids in the cholesterol-fed rabbit this agent was without effect on endogenous cholesterol metabolism in this species. These findings are unlike those observed in man in whom significant decreases of serum lipids have been noted following oral administration of neomycin(1). The greater degree of aortic atherosclerosis and visceral lipoidosis resulting from oral administration of neomycin to cholesterol-fed rabbits appears related to the greater degree of hypercholesterolemia in these animals. Other factors noted to influence cholesterol atherosclerosis in the rabbit such as undernutrition, hypertension or alteration of the cholesterol:phospholipid ratio were not apparent. Although the exact mechanism responsible for the effects of neomycin in the rabbit is inapparent it appears reasonable to assume that it is related to increase in intestinal absorption of cholesterol. Intramuscular injections of neomycin failed to alter degrees of lipemia or aortic atherosclerosis in treated cholesterol-fed rabbits from that observed in cholesterol-fed controls. It appears significant that orally administered neomycin is highly stable in the intestinal tract and is almost totally (97%) eliminated by this route, whereas that following intramuscular injection is excreted in the urine. Whether the accentuating effect of orally administered neomycin on cholesterol atherosclerosis is related to depression of intestinal bacterial flora resulting from administration of this drug remains to be demonstrated.

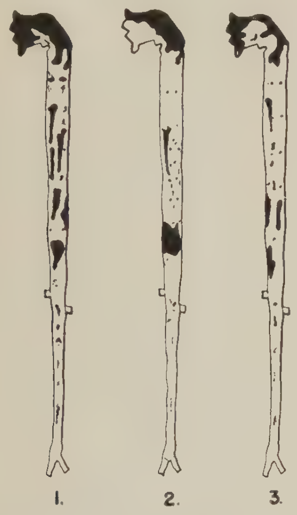

FIG. 1. Schematic representation of degree and distribution of aortic atherosclerosis. 1. oral neomycin, cholesterol-fed; 2. untreated, cholesterol-fed; 3. intramusc. neomycin, cholesterol-fed.

Summary. Prolonged oral administration of neomycin resulted in a greater elevation of serum lipids and more severe aortic atherosclerosis and visceral lipoidosis in cholesterol-fed rabbits than in untreated cholesterol-fed controls. Failure to observe these enhancing effects following intramuscular administration of neomycin indicates that this action is related to increased absorption of cholesterol. Neomycin was without effect on endogenous cholesterol metabolism in this species.

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Peripheral Metabolism of Thyroxine and Triiodothyronine in Iodine Deficient Rat.*† (25697)

JOSEPH D. FELDMAN (Introduced by F. J. Dixon)
Dept. of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pa.

In the iodine deficient rat, iodide trapping activity and rate of hormonal secretion by thyroid are markedly enhanced(1). Morphologic and metabolic aspects of the gland indicate extreme activity while there is insufficient output of hormones. The present experiments were therefore undertaken to determine whether the iodine deficient state influences peripheral disposition of thyroidal hormones, more particularly, rate of disappearance from serum of radiothyroxin (T4*) and radiotriiodothyronine (T3*), their space of distribution and quantity of hormone degraded daily.

Materials and methods. In each experiment, 14 male rats were divided into 2 groups of 7 each. One group was placed on iodine deficient diet‡ and distilled water for 5 weeks; the control group received tap water and pellet diet containing 43 μg of KI/100 g of diet. Then, all rats were injected subcutaneously with 50 mg of propylthiouracil (PTU) in 0.5 ml of saline and 15 minutes later *via* tail vein with about 20 μc of radiohormone.§. They were bled from tail vein at 6 and 12 hours and every 12 hours thereafter until autopsy, at which time they were exsanguinated. For T4*, period of observation lasted 72 hours, and for T3* 48 hours. Urines and feces were collected for each 24 hour period. At autopsy, thyroids were removed, cleaned of adventitial tissue, and weighed on torsion balance to the nearest 0.1 mg. Radioactivity in thyroids, urine, feces and whole carcass (minus blood and thyroids) was counted. A measured aliquot of serum from each bleeding was counted for total and Somogyi precipitable radioactivity. Half-lives of radiohormones were determined by inspection of the disappearance curves, constructed on semi-log paper plotting serum radioactivity against time in hours. Space of distribution was calculated from total radioactivity injected, divided by counts/min/ml of serum at zero time, the latter figure obtained by extrapolating the straight line of disappearance curve to the ordinate axis. Chromatograms were made from extracts of each bleeding and from each 24 hour urine collection. One ml of serum from each animal of a group was pooled for each bleeding. Pooled sera and a mixture of mono-iodotyrosine, diiodotyrosine, triiodothyronine and thyroxine were prepared for chromatography as previously described(1), using butanol-acetic and water-collidine-ammonia systems. NaI solution was run separately in the same chromatocab at the same time. Spots were developed with sulfanilic diazonium chloride and palladium chloride for iodide ion. The chromatogram was divided into 1 cm wide strips and each strip counted. The spots were then removed and counted. Urines for each 24 hours were handled in the same fashion. Serum I^{127} determinations were done on samples taken from rats at autopsy.‖

Results. Disappearance of radioactivity from serum of rats given PTU and T4* was linear over 72 hours. The half life of hormone was approximately 18 hours in both iodine deficient and control groups of animals. In Fig.

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‡ Nutritional Biochemicals Corp., Cleveland, O.

§ Radiothyroxine and radiotriiodothyronine were supplied by Abbott Labs., Oak Ridge, Tenn.

‖ Performed in laboratory of Dr. J. Sugarman, Pittsburgh, Pa.

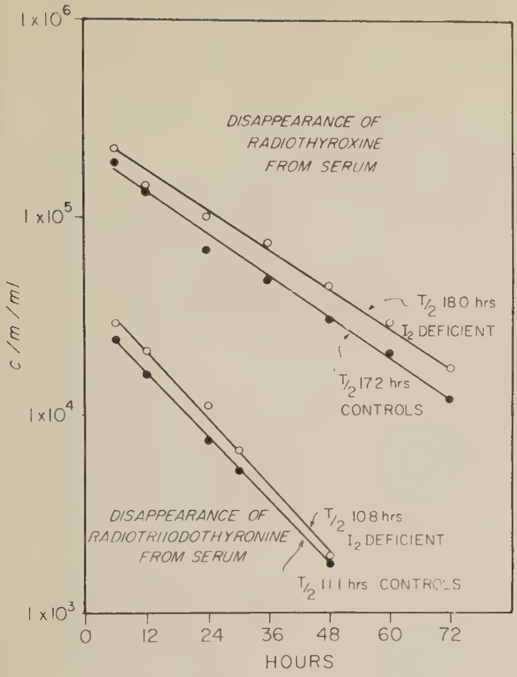

FIG. 1. Disappearance curve of radiothyroxine and radiotriiodothyronine from the serum of iodine-deficient and control rats.

1, curves were constructed from averages of each group, representing protein bound I^{131}/ml of serum. Within 6 hours of intravenous injection, 70-75% of circulating I^{131} was precipitable. The precipitable I^{131} remained at this level 48 hours, then gradually dropped to 65% at 72 hours. By chromatography, T4* and I^{131} iodide were found, most radioactivity being in the T4* spot.

It was necessary to inject PTU to obtain a straight line disappearance curve of serum I^{131}. In other experiments without PTU, recycling of I^{131} was sufficiently rapid, especially in iodine deficient rats, that an exponential decay curve was not observed. The efficacy of PTU was demonstrated by thyroidal uptake of I^{131} in iodine deficient rats after 3 days of 0.25% of the radioactive dose given or 50-100 times less than uptake in deficient rats without PTU. It is also to be noted that the iodine deficient thyroid concentrated about 5 X more I^{131} than its control.

The space of distribution of T4* was similar in both experimental and control rats, being 76 ml or 17.3% of body weight and 84 ml or 18.4% of body weight for the 2 groups respectively. PBI^{127} levels in iodine deficient and control groups averaged 2.7 μ and 4.0% respectively (Table II).

From above data of turnover rate, space of distribution and I^{127} level, quantity of thyroxine degraded daily was calculated, assuming that 4/5 of the PBI^{127} was thyroxine. The iodine deficient group of rats degraded daily much less thyroxine than did their controls.

Both renal and fecal excretion of I^{131} were less in deficient rats than in controls, despite the fact that the thyroid was excluded from the metabolic pool. These differences in excretion and retention between control and experimental rats were not statistically significant because of wide spread of values from animal to animal.

Results similar to those just recorded for T4* were obtained in iodine deficient and control rats following administration of PTU and T3*. The half life was between 10.5 to 11 hours for the 2 groups of animals (Fig. 1). The curves represent averages of protein bound radioactivity/ml of serum. Within 6 hours of intravenous injection of T3*, only 50-60% of circulating radioactivity was protein bound, and this was T3* by chromatography. At 48 hours, PBI^{131} decreased between 10-20% of circulating radioactivity.

Space of distribution was of the same dimension for both deficient and control groups (Table II). PBI^{127} averaged 1.5μ% in experimental rats compared with 2.2μ% in controls. If one estimated circulating T3 to be 1/5 of total circulating PBI^{127}, then again the quantity of T3 degraded daily would be less in deficient rats than in controls (Table II).

Discussion. In the iodine deficient state, despite markedly augmented activity of the thyroid and the accelerated cycling of iodine compounds, peripheral metabolism of T4* and T3* remained unaffected. Turnover rates or half lives observed in iodine deficient groups were of the same order as for controls and as was found by others(2-6). Nor was there an alteration of space of distribution resulting from iodine deficiency. There was, however, a diminution in quantity of hormone degraded daily in the deficient group, simply because of inedaquate secretion of thyroid hormones. In short, the iodine deficient rat had less thy-

TABLE I. Excretion, Retention and Thyroidal Uptake of I^{131} in Iodine Deficient Rats Following Injection of PTU and T4* or T3*.

Animal	Body wt, g	Thyroid wt, mg	Thyroid wt, % of body wt	Thyroid uptake, % of dose	Urine excretion, % of dose	Fecal excretion, % of dose
T4* + PTU						
I_2 deficient	436	*29.2 ± .70**	*6.7 ± .4*	*.25 ± .027*	16.0 ± 1.17	35.4 ± 5.3
Controls	455	18.1 ± 1.21	4.0 ± .15	.03 ± .007	22.9 ± 3.2	47.8 ± 2.6
T3* + PTU						
I_2 deficient	468	*30.1 ± 3.45*	*6.4 ± .55*	*.34 ± .06*	28.9 ± 1.5	46.5 ± 7.1
Controls	420	19.2 ± 1.01	4.6 ± .34	.07 ± .008	40.0 ± 4.5	35.5 ± 4.7

* ± stand. error of mean.

Italicized figures are significantly different from controls at 1% level or less.

TABLE II. Half-Life, Space of Distribution and Daily Degradation of Thyroidal Hormones in Iodine-Deficient and Control Rats.

Animal	Half-life hours	Daily turnover rate	Space of distribution, ml	Serum I^{127}, μ %	Daily degradation,* μ
T4* + PTU					
I_2 deficient	18.6	.89	76	2.7	1.46
Controls	18.2	.91	84	4.0	2.44
T3* + PTU					
I_2 deficient	10.3	1.65	300	1.5	1.49
Controls	11.0	1.50	389	2.2	2.57

* Assuming ⅘ of serum PBI^{127} is T4 and ⅕ is T3.

roidal hormone than its control, but metabolised whatever was available at the same rate as the control.

Summary. The peripheral metabolism of radiothyroxine (T4*) and radiotriiodothyronine (T3*) was studied in iodine deficient and control rats. The half-life and space of distribution of these hormones were similar in the 2 groups of animals. The quantity of hormone degraded daily, however, was less in iodine deficient than in its control rat because less was produced.

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Lipid Synthesis by Ascites Tumor Cells.* (25698)

DOROTHY L. FILLERUP, JAMES W. KNAUF AND JAMES F. MEAD

Laboratory of Nuclear Medicine and Radiation Biology, Dept. of Biophysics and Nuclear Medicine, University of California, Los Angeles

We have shown(1) that palmitic acid is readily absorbed by Ehrlich mouse ascites tumor cells *in vitro*, especially when presented as the albumin complex. Moreover, an average of about 40% of the absorbed palmitic acid was oxidized to CO_2 per hour under these conditions. However, it was not ascertained

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* These studies were supported by contract between Atomic Energy Commission and Univ. of California.

whether the remainder of the absorbed lipid was used for synthesis of cellular lipids or whether these could be absorbed from the medium. A preliminary experiment revealed that when tumor cells are exposed to labeled triglyceride present in the medium as low-density lipoprotein, oxidation of fatty acid does not occur, indicating that triglyceride is probably not absorbed.† Therefore, experiments were initiated to determine, first, if the various cellular lipids are in fact synthesized by these cells from absorbed fatty acids and at what rate, and second, under what conditions, if any, these newly synthesized lipids appear in the medium.

Methods. Treatment of tumor cells. Ehrlich ascites tumor cells and ascitic fluid were obtained as described previously(1). In each of the experiments, 4 mice were used, yielding a total of 45-50 ml of blood-free tumor. The tumor was separated by centrifugation into ascitic fluid (about 30 ml), which was retained for use as the incubation medium, and cells, which were washed twice with buffer. To obtain tumor cells containing palmitic-1-C^{14} acid and its metabolic products for incubation in a C^{14}-free medium, the cells were suspended in 30 ml of ascitic fluid in a 100 ml round-bottom flask and were incubated at 37° in air with 5 ml of an albumin-palmitate-1-C^{14} solution containing approximately 1.1 mg palmitic acid of specific activity 1.7×10^5 d.p.s. per mg for a total of 1.9×10^5 d.p.s. After 60 minutes, the cells were separated by centrifugation at 1300 x g and were washed twice with phosphate buffer. These washed cells, containing palmitic-1-C^{14} acid and its metabolic products were then reincubated with fresh ascitic fluid for 10, 20, 30, and 60 minute intervals, following which the cells were separated from the medium by centrifugation and lipids from both the cell and supernatant fractions were extracted as described previously(1). In an attempt to study a "0" time, the tagged cells were placed in 30 ml of fresh ascitic fluid, as in the case of the other timed experiments, but were immediately centrifuged to separate them from the medium, the entire procedure being carried out at 0°C. In one experiment, a portion of the cells, labeled with palmitate-1-C^{14}, were incubated with buffer and the remainder with ascitic fluid for 60 minutes to determine whether the nature of the medium might influence passage of lipids from the cells. *Separation of lipids.* Each sample of extracted lipid was separated by chromatography on silicic acid as described previously(1, 2). In view of the high activity of the added palmitate-1-C^{14} and the possibility of misinterpretation of small amounts of contamination of other lipid fractions, each fraction was further purified. Fraction 1, which was eluted with 1% ether in petroleum ether (normally the sterol ester fraction) was passed through a short alumina column in chloroform to ensure absence of any free fatty acid. Fraction 2, eluted with 5% ether in petroleum ether (normally the triglyceride fraction), fraction 4, eluted with 25% ether in petroleum ether (normally diglyceride) and fraction 5 eluted with ether (normally monoglyceride), were passed through short alumina columns with ether:ethanol (1:1).‡ Fraction 3, eluted with 10% ether in petroleum ether, (normally cholesterol and free fatty acids) was rechromatographed on silicic acid using 5%, 8% and 10% ether in petroleum ether as eluants. The last 2 fractions were then passed through a short alumina column in chloroform. The final fraction removed from silicic acid with methanol was purified by precipitation with acetone. Plating and counting procedures have been described(1). All counting was performed with a Nuclear Instruments and Chemical Corp. D-47 Micromil Flow Counter.

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† One ml of ascitic fluid from a mouse inoculated with tumor 7-9 days previously, was emulsified with 10 mg of triolein transesterified with palmitic-1-C^{14} acid. The emulsion was reinjected intraperitoneally into the mouse and, after 5 minutes, the tumor was withdrawn and ascitic fluid separated by centrifugation into lipoprotein and albumin fractions. The low-density lipoprotein fraction was incubated 60 minutes with tumor cells from different mice as described previously(1). Results of 3 experiments revealed no significant activity in the center well, with activity distributed about equally between cells and medium.

‡ It is essential that the alumina columns are short and that glycerides are in contact with the alumina for as short a time as possible in order to avoid hydrolysis.

TABLE I. % of Recovered Activity in Cells and Supernatant Solution Following 60-Minute Incubation of Washed Cells Previously Labeled with Palmitic-1-C^{14} Acid.

Medium	% admin. activity in washed cells	% recovered activity in:	
		Cells	Supernate
Buffer	17.8	93.6	6.4
Ascitic fluid	19.3	90.9	9.1

Results. Table I presents results of experiment to determine whether the nature of the medium influences passage of lipids out of cells previously incubated with palmitate-1-C^{14}. Although most of the activity remained in the cells, it is apparent that there is little or no difference in the amount appearing in the medium whether it be ascitic fluid or buffer. This result is quite different from that found by James, Lovelock and Webb(3) for the erythrocyte, in which synthesized lipid is readily exchanged with the medium only in presence of α-lipoproteins.

Table II summarizes mean relative specific activity and per cent total activities of the purified cell lipids at times from 0 to 60 minutes. After one hour's incubation with the palmitic acid, phospholipid synthesis is still progressing at the expense of free fatty acid, which declines steadily throughout the experimental period. Triglyceride synthesis is also taking place throughout the experiment, possibly at the expense of both free fatty acid and phospholipid. Synthesis of the other lipids is much slower, but apparently continues during the 60-minute period.

Table III summarizes mean relative specific activities and per cent total activities of the lipids from the incubation media at times from 0 to 60 minutes. Weights of the lipid fractions from the incubation media were so low that accurate determination was difficult, especially in the case of the di- and monoglycerides. Moreover, very little of the cell activity is released to the medium during 60 minutes (Table I).

Most of this is in the free fatty acid fraction, which may possibly have been bound loosely to the cell wall rather than within the cell, and therefore resisted the washing process and was slowly removed during incubation. The major synthesized lipid fraction, phospholipid, is also the major fraction released to the medium. Triglyceride is apparently retained within the cell.

Discussion. It has been shown(4,5,6) that neoplastic cells carry on an active, if not exclusive oxidation of fat as the normal energy source. Absorption of fatty acid may be, therefore, an important process for such cells, especially since it is possible that they may not be capable of fatty acid synthesis. The fatty acid absorbed by the cell is partially oxidized and partially converted into the various cell lipids (largely phospholipid with smaller amounts of triglyceride). The process of synthesis from fatty acid is apparent from its steady decline in the cells and the accompanying increase in the other fractions. The exact precursor-product relationships are, however, not revealed and are obviously too complex for a simple conversion mechanism. The decline of phospholipid and its possible participation in synthesis of other lipids are of interest.

Of the major lipids synthesized by the cell, only phospholipid is released into the medium

TABLE II. Activities of Lipid Fractions from Ascites Tumor Cells Following Incubation with Palmitic-1-C^{14} Acid.

Fraction	0 min.†	10 min.†	20 min.†	30 min.†	60 min.‡
Sterol ester	.1§ (.3)‖	.3 (1.1)	1.2 (2.0)	1.0 (2.2)	8.5 (5.3)
Triglyceride	.4 (.8)	5.1 (13.0)	4.9 (14.6)	4.6 (15.4)	4.1 (19.7)
Fatty acid*	91.6 (51.7)	87.3 (34.8)	80.8 (24.8)	81.0 (20.3)	70.8 (12.5)
Sterol	.1 (.2)	.3 (1.0)	.4 (.8)	.8 (1.9)	.2 (.1)
Diglyceride	.2 (.3)	.8 (1.8)	1.2 (2.1)	1.6 (2.9)	1.9 (4.1)
Monoglyceride	.1 (.1)	.2 (.8)	.3 (1.2)	.5 (2.1)	7.0 (9.0)
Phospholipid	7.5 (46.7)	6.0 (47.5)	11.2 (54.4)	10.5 (55.2)	7.5 (49.4)

* Calculated from weight and activity retained on alumina column.

† Means of 3 determinations.

‡ " " 2 " .

§ Relative specific activity (specific activity of fraction/sum of specific activities).

‖ Values in parentheses represent % of total activity.

TABLE III. Activities of Lipid Fractions from Incubation Media of Ascites Tumor Cells Which Had Been Incubated with Palmitic-1-C^{14} Acid.

Fraction	0 min.[†]	10 min.[†]	20 min.[†]	30 min.[†]	60 min.[‡]
Sterol ester	.1[§] (.3)[‖]	— (.2)	.1 (.2)	.1 (.4)	.8 (1.9)
Triglyceride	.1 (.6)	.1 (.7)	— (.5)	.1 (.8)	.4 (2.0)
Fatty acid[*]	97.7 (85.1)	98.4 (86.3)	97.0 (78.3)	96.5 (78.7)	71.6 (30.5)
Sterol	.3 (3.2)	.1 (1.6)	.5 (3.3)	.2 (2.2)	2.8 (3.5)
Diglyceride	.1 (.8)	.1 (1.2)	.3 (1.5)	.2 (2.2)	8.4[¶] (19.9)
Monoglyceride	.2 (1.1)	.1 (1.4)	— (.4)	.1 (1.1)	9.6[¶] (10.4)
Phospholipid	1.5 (8.9)	1.1 (8.6)	2.2 (15.8)	2.9 (14.6)	6.4 (31.9)

[*] Calculated from weight and activity retained on alumina column.

[†] Means of 3 determinations.

[‡] Only 1 determination.

[§] Relative specific activity (specific activity of fraction/sum of specific activities).

[‖] Values in parentheses represent % of total activity.

[¶] Values are probably not reliable because of extremely small amount of material (less than 1 mg) in each case.

to any extent. Triglyceride does not appear to be either absorbed or released and sterol ester may behave similarly. Thus, it is possible that only the more polar lipids can readily pass through the cell wall.

Further studies with labeled lipids should serve to amplify these indications.

Summary. Ehrlich ascites tumor cells were incubated in ascitic fluid with albumin-palmitate-1-C^{14} complex and were then washed and incubated for varying time intervals with fresh media. Lipids of both cells and media were extracted, separated chromatographically and counted. Results show a decrease of cell free fatty acid with increases in all lipids, especially triglyceride. The major lipid synthesized is phospholipid, which, however declines after 30 minutes. Only phospholipid is released into the medium to any extent.

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Stimulation of Neurosecretion in Shark Embryos by Thyroid Hormones.[*]

(25699)

AUBREY GORBMAN AND SUSUMU ISHII[†]
Barnard College, Columbia University, N.Y.

The principal physiological role of the hypothalamic neurosecretory system appears to be integration of pituitary gland with certain environmental changes(1). Since this integrative mechanism is involved in such phenomena as seasonal breeding it is not surprising that it is known to differentiate relatively late in the life cycle. In both rats(2) and teleost fishes(3) such differentiation, physiologically and morphologically, appears incomplete until post-embryonic life. Recently, while studying morphological features of the hypothalamic neurosecretory apparatus of shark (*Squalus suckleyi*) embryos, we found that its differentiation is hastened by treatment with thyroid hormones. Since nothing now is known of factors which influence development and differentiation of this important system, it seemed to us worthwhile to report this fact apart from the detailed anatomical description.

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[†] Present address: Zoolog. Inst., Tokyo Univ., Japan.

Methods. About 75 "pup" embryos (length 185 to 210 mm) and about 30 "candle" embryos (length, 61 to 69 mm) of the ovoviviparous shark, *Squalus suckleyi* were removed from uteri of females caught at Friday Harbor, Wash., in Aug., 1958, and kept in glass bowls in groups of 4 or 5 in slowly flowing sea water at 12°-14°C. The embryos of this species spend approximately 2 years *in utero*. The "pups" used were within a month of parturition, and the "candle" embryos about 13 months. Each in a group of 4-5 embryos in one bowl received the same treatment, consisting of intraperitoneal injections of 0.05 cc of 0.8% NaCl solution (control), or this volume of salt solution containing 50 μg propyl thiouracil, 10 μg L-thyroxine, 100 μg L-thyroxine, 100 μg triiodothyronine, 10 μg triiodothyroacetic acid or 100 μg triiodothyroacetic acid. Animals were injected every other day until killed after 2 or 3 weeks of such treatment. Brains of embryos were fixed in Bouin's fluid and serial sections stained with Gomori's chromalum haematoxylin-phloxine (CHP), or aldehyde fuchsin (AF), or by periodic acid-Schiff reaction (PAS).

Results and Discussion. In almost "full-term" embryos a single pair of small neurosecretory centers was found, the preoptic nuclei. Cell bodies of constituent neurones contained very fine granules stainable with all 3 staining procedures. Axones from bilateral centers, also containing stainable neurosecretory granules extended as a preoptico-hypophyseal tract into the neurohypophysis. In the neurohypophysis numerous endings of the secretory axones stained strongly with various stains showing accumulations of stainable material, particularly adjacent to small blood vessels. There was no clear difference in apparent amount of neurosecretion, or in its pattern of distribution between control embryos and those treated with thyroid hormones.

In younger "candle" control embryos no stainable neurosecretory material was discernible in the preoptic nuclear area, or in any of the axones between this area and the neurohypophysis. In the neurohypophysis careful study revealed only sparsely distributed droplets of neurosecretion in the endings of axones. In embryos treated with any of the thyroid hormones there was in almost every instance an increase in number of AF-stained droplets in the neurohypophysis; in most cases numbers of such droplets were more than doubled. An even more striking change in thyroid-treated candle embryos was in the appearance of numerous small droplets in length of the preoptico-hypophyseal neurosecretory tract, running posteriorly in the infundibular floor, above the prolonged tubular adenohypophysis. There was no corresponding change in the preoptic nuclear area. Histological study made of the skin, digestive tract, and respiratory organs of the thyroid hormone treated candle stage embryos revealed no difference from control or propyl thiouracil treated specimens.

It is clear that in the pituitary and hypothalamus of the candle stage shark embryo only the first signs of preoptic nuclear neurosecretory activity can be detected. Between this stage and the "near-term" pup embryo an important degree of differentiation in this system occurs, both quantitatively and qualitatively. Treatment of younger embryos with thyroxine, triiodothyronine, or triiodothyroacetic acid accelerated the differentiation of the hypothalamic neurosecretory apparatus.

Although thyroid hormone has not been known before to affect differentiation of hypothalamic neurosecretion, it is well known as a stimulant of structure and functional differentiation in other parts of brains of amphibian tadpoles and young mammals(4,5,6). The interesting possibility raised by these findings is that thyroxine may also be a stimulant of the adult hypothalamic neurosecretory system. If so, this would explain at least partially, the synergism between thyroid hormone and such adenohypophysis-controlled phenomena as growth and gonadal development.

Summary. In embryos of the shark, *Squalus suckleyi,* differentiation of hypothalamic neurosecretory system is accelerated by treatment with thyroxine, triiodothyronine or

triiodothyroacetic acid. Early accumulation of stainable neurosecretory granules is stimulated in the preoptico-hypophyseal fibre tract, and in the neurohypophysis.

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Further Studies on Passive Transfer of Protection Against Lethality of Endotoxin (25700)

HENRY H. FREEDMAN (Introduced by A. S. Gordon)
Princeton Labs., Princeton, N. J.

We have recently described passive transfer, by serum or plasma of endotoxin-tolerant rabbits, of protection against lethality of homologous and heterologous endotoxin in the mouse, and of tolerance to pyrogenicity of endotoxin in the rabbit(1,2). Passive transfer from no-longer tolerant donors, after reticuloendothelial system blockade, and failure to obtain protection when tolerant serum and endotoxin challenge are mixed and given as a single injection, were also demonstrated (2,3). These findings argue against mediation of tolerance by antibody, circulating or fixed. Like endotoxin tolerance itself, transfer appears related to the demonstrated stimulation of RES of recipient(3). Investigations to identify the humoral mediator have eliminated certain obvious possibilities. The present report describes experiments ruling out adrenal cortical hormone, properdin, and phagocytosis-promoting factor. Studies showing protection afforded by normal donor serum under limited experimental conditions, and effect of heat treatment of donor blood are included.

Materials and methods. Preparation and treatment of donor rabbits and recipient mice, and precautions against pyrogen contamination, were as previously described(1,2). Endotoxins, Difco lipopolysaccharides of *S. typhosa* 0901 and *E. coli* 0127:B8, were given *i.p.* for challenge. Aliquots of donor serum were adjusted to pH 5.5 with N HCl, placed into boiling water bath 5 minutes, filtered, and the coagulum washed with saline. The combined filtrate was brought to original serum volume and adjusted to pH 7 with N NaOH. Sera were treated with zymosan (Mann Res. Labs, Lot #4393) both for preparation of "RPb" by 2-stage adsorption at 17 and 37°C, and by exhaustive exposure at 37°C which promotes destruction of C′3, and the zymosan eluted for properdin(4,5). Hydrocortisone was given *i.p.* in 0.2 ml volume at 1 and 5 mg 3 hours before challenge. Adrenal weight was determined for normal and endotoxin-tolerant donor rabbits at sacrifice. Corticosterone was estimated in extracts of normal and tolerant donor plasma by absorption in methanol at 240 mμ. Possible appearance of 17-OH corticoid from stimulation of pituitary-adrenal axis by endotoxin treatment for inducing tolerance was measured by Porter-Silber method(6). Phagocytosis-promoting factor (PPF) of serum was adsorbed on $BaSO_4$ and eluted with citrate(7). In all instances serum or plasma was given *i.p.* in 1 ml volume. Schedules for treatments and challenge are given in the Tables.

Results. The data eliminating adrenal cortical hormone, properdin, and PPF, as mediators of the protective effect of endotoxin-tolerant donor blood administered 3 hours before endotoxin challenge are given in Table I. Steroid, in several thousand times the content of donor plasma (<0.5 μg/ml), did not protect in these experiments. Mean adrenal weight for 19 endotoxin-tolerant rabbits, ob-

TABLE I. Failure of Adrenal Cortical Hormone, Properdin, or Phagocytosis-Promoting Factor to Account for Protection against Endotoxin Lethality in Mice by *S. typhosa* Endotoxin-Tolerant Rabbit Serum.

Group*	No. exp.	Dead/Total	% dead
Controls (LD_{75})	5	40/50	80
Hydrocortisone, 1 mg	2	13/20	65
" , 5 "	1	7/10	70
Tolerant donor serum	3	6/30	20†
Idem, zymosan-adsorbed‡	1	1/10	10†
" , " " §	2	5/20	25†
" , $BaSO_4$ "	1	2/10	20†

* Treatments given *i.p.* 3 hr before *i.p.* LD_{75} *S. typhosa* endotoxin.

† $p < .001$.

‡ 2-stage adsorption, 17° then 37°C.

§ 37°C adsorption, 1 hr.

tained 24 hours after the last of 6 daily injections of endotoxin, was 183 mg, and for 15 normal rabbits was 187 mg. No difference in total steroid absorbing at 240 mμ between normal and tolerant rabbit plasma was found, and no evidence for appearance of Porter-Silber reacting steroid was obtained. Zymosan-adsorbed tolerant serum retained its potency; it is worth noting that from preparation of RPb the zymosan eluate, which did not give protection, contained protein (280 mμ absorption) many times the total properdin content of serum. Adsorption of tolerant serum with $BaSO_4$, for removal of PPF, was also without effect; the eluate did not protect.

The limited protection afforded by normal donor serum or plasma, when given 30 minutes before homologous or heterologous endotoxin, is shown in Table II. Degree of protection given by tolerant donor blood exceeded that obtained with normal. Heating completely removed activity from normal serum, but not, apparently, from tolerant. With neither endotoxin challenge did the mixture of normal serum and endotoxin, prepared just before injection, give protection.

Discussion. Clearly, adrenal cortical hormone content of tolerant donor blood is not responsible for protection against endotoxin lethality. This is not surprising; large amounts of steroid, given close to time of endotoxin challenge, are required for protection (8,9). No evidence was obtained for stimulation of the pituitary-adrenal axis by treatment used to induce endotoxin tolerance in donor rabbits. Tauber and Garson(10) have shown protection against endotoxin lethality in mice by properdin, when the latter was mixed with the toxin and given as a single *i.p.* injection. This required large amounts of properdin, and brings to mind the ability of endotoxin, like zymosan, to bind with properdin(5,11). The protection seen following passive transfer from tolerant donor in our system, not obtained with simultaneous administration(3), is not attributable to properdin content of donor blood. Although a single dose of endotoxin produces alterations of serum properdin(12), the endotoxin-tolerant rabbit(13) and rat(14) had normal properdin titers, indicating development of tolerance to this response. From known effects of endotoxin on leucocytes, PPF was considered; this too is eliminated. Studies on a lipid fraction, isolated from tolerant donor blood, which stimulates RES of normal mice, and protects them against lethality of endotoxin, will be published later.

Limited protection afforded by normal serum is obtained neither when an interval of 3 hours between injections is allowed nor when the challenge is given *i.p.* 30 minutes after *i.v.* administration of normal serum, in contrast with passive transfer from tolerant donor which protects substantially in both circumstances(1). Inactivation of endotoxin by direct reaction with a factor present in

TABLE II. Limited Protection Against Endotoxin Lethality in Mice by Normal Rabbit Serum Given *i.p.* 30 Minutes Before *i.p.* Endotoxin Challenge.

Exp. and group	No. exp.	Dead/Total	% dead
S. typhosa challenge (LD_{75})			
Controls	5	38/46	83
Normal donor serum	2	5/18	28*
Idem, heated	2	16/18	89
Tolerant donor serum	2	0/18	0*
Idem, heated	2	10/18	56
Mixture, normal serum-endotoxin†	1	8/10	80
E. coli challenge (LD_{85})			
Controls	3	25/28	89
Normal donor serum	2	7/20	35*
Mixture, normal serum-endotoxin†	1	8/10	80

* $p < .001$.

† Serum and endotoxin challenge mixed and immediately injected *i.p.*

normal serum seems most unlikely. The "endotoxin-detoxifying component" of normal blood found by Landy and associates(15) and active *in vitro,* can hardly explain our *in vivo* findings in view of the full lethality seen after simultaneous administration of serum and endotoxin extemporaneously mixed. The results suggest need for time for a recipient animal response, and failure with the *i.v.* route with the same 30 minute interval points to an intraperitoneal mechanism. Results with heat-treated serum, to yield a relatively protein-free filtrate, must be interpreted with caution. The protein coagulum formed may, despite washing, trap much non-protein. The results with tolerant donor serum suggest a non-protein moiety. Processing of normal and endotoxin-tolerant donor serum and plasma by the phenol-water partition method for lipopolysaccharide has not yielded endotoxin activity (unpublished experiments).

Summary. The broad protection against lethality of endotoxin obtained by passive transfer from endotoxin-tolerant donors is not mediated by adrenal cortical hormone, properdin, or phagocytosis-promoting factor. Limited protection by normal donor serum given *i.p.* shortly before, but not simultaneously with, inoculation of endotoxin has been demonstrated. The results are not explained by direct inactivation of endotoxin by serum. Heating removes this activity from normal, but not completely from tolerant, donor serum.

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Effect of Tumor Cell Fractions upon Plasma Iron, Liver Catalase and Organ Weights of Rats. (25701)

Ralph F. Kampschmidt and Thomas A. McCoy
Biomedical Division, Samuel Roberts Noble Fn., Ardmore, Okla.

A heat stable, water soluble, and alcohol precipitable extract from many different types of tumors contained a factor(s) which depresses liver catalase activity when injected into normal mice(1). Nakagawa and Nakagawa(2) reported that centrifugal fractionation of homogenates of Yoshida sarcoma and ascites hepatoma, yields an active supernatant fraction, which contained soluble protein and microsomes. The nuclear or mitochondrial fraction failed to show clear evidence of liver catalase depressing action. Our previous studies(3,4) indicated that an extract from Walker carcinosarcoma 256 or Flexner-Jobling carcinoma depresses hepatic catalase activity, lowered plasma iron and affected the weights of liver, spleen and thymus. Homogenates of the Walker tumor were, therefore, fractionated by centrifugation to separate the

TABLE I. Distribution of Factor which Depresses Plasma Iron of Normal Rats in Fractions Isolated from Homogenates of Walker Carcinosarcoma 256

	Dry wt of injected material (mg)				
Tumor fraction	.5	1.	2.5	5.	10.
			Plasma iron μg %		
Sham*	268 ± 13†	273 ± 7	263 ± 27	270 ± 11	259 ± 17
Tumor homogenate			175 ± 14	131 ± 17	120 ± 14
N_W‡			203 ± 27	222 ± 24	179 ± 16
M_{W2}	152 ± 24	107 ± 21	77 ± 14	58 ± 5	66 ± 8
P_W			195 ± 30	216 ± 41	170 ± 9
S_2			251 ± 18	248 ± 31	204 ± 6

* Vehicle only.

† Each figure represents mean value for 4 rats ± stand. error (with duplicate determinations on each rat).

‡ For explanation of fraction designations, see text.

active substance(s) that produced these many diverse effects.

Methods. Homogenates of Walker carcinosarcoma 256 were fractionated in 0.25 M sucrose according to method outlined for liver by Schneider and Hogeboom(5). Fraction designations and centrifugation speeds used were as follows: NW-nuclear fraction (10 min at 700 X G), MW_2 – mitochondria fraction (10 min at 5000 X G), S_1 – supernatant and washings from mitochondrial fraction, P_W – submicroscopic particles (35 min at 100,000 X G), S_2 – supernatant and washing from submicroscopic particles. Particulates were suspended in 0.25 M sucrose and injected intraperitoneally into Holtzman rats weighing 160-180 g. Methods to determine plasma iron concentrations and liver catalase activity have been described(3,4). Determinations of plasma iron concentration were made 16 hours after administration of various fractions, whereas all other measurements were conducted 48 hours later.

Results. The effect of various fractions of the Walker tumor upon plasma iron of normal rats is shown in Table I. Mitochondria consistently produced the greatest depression in plasma iron. This fraction contained 8.7 ± 0.4% of total dry weight of the tumor. The mitochondrial fraction, even at 10-fold dilution, depressed plasma iron to essentially the same level as homogenate. It was therefore concluded that most of the activity was present in this fraction.

Since it was possible that only plasma iron was affected by injections of mitochondrial fractions, effects of tumor cell fractions upon liver, spleen, and thymus weights and liver catalase activity were determined (Table II). Only 25 mg of the various fractions was injected. It can be seen that the mitochondrial fraction significantly increased weights of liver and spleen and depressed thymus weight and catalase activity. While some of these effects were produced by other tumor fractions, mitochondria injections consistently produced the largest changes.

Discussion. It was shown that homogenates of tumor produce changes in organ weights, plasma iron and liver catalase at much lower concentrations than normal tissue homogenates(4). These changes, moreover, resembled those which occurred during early growth of the tumor. The present results again raise

TABLE II. Effects of Various Tumor Fractions upon Organ Weights and Liver Catalase Activity in Normal Rats.*

	Tumor fraction			
	Sham	N_W	M_{W2}	S_1
No. rats	8	8	8	8
Liver†	1180 ± 30‡	1240 ± 30	1320 ± 20§	1200 ± 30
Spleen†	75 ± 2	134 ± 10§	144 ± 6§	114 ± 8§
Thymus†	64 ± 3	55 ± 4	42 ± 3§	60 ± 5
Liver catalase‖	5470 ±230	3730 ±240§	2700 ±180§	4250 ±240§

* Intraper. injections of 25 mg dry wt/rat. † mg dry wt/100 g body wt. ‡ Mean ± stand. error. § Significantly different from sham ($P < .01$). ‖ Catalase units(4).

the question, however, whether or not a substance present in tumor, such as the presently reported active material in mitochondria, can enter the general circulation of host(1). Nakahara and Fukuoka found that freshly prepared extracts of tumor tissue contained a small amount of dialyzable substance which depresses liver catalase of normal mice(6). In our experiments permitting mitochondria to stand several hours in 0.25 M sucrose at 0°C and then centrifuging, resulted in high proportion of activity in the supernatant.

The discrepancy between present results and those previously reported by Nakagawa and Nakagawa(2) may be due to different tumors employed or possibly to leakage of active substance(s) from mitochondria. Nakagawa, *et al.*(7) to purify toxohormone, found a highly active nucleic acid fraction. Investigations by other groups indicated that the active material may be a polypeptide(8). It is very unlikely that the active material in present studies was in the nucleic acid fraction, since it was concentrated in mitochondria.

Summary. The factor(s) in Walker carcinosarcoma 256 tissue which caused a depression in plasma iron, liver catalase activity and alterations in organ weights when injected into normal rats was localized primarily in the mitochondrial fraction.

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Norepinephrine Depletion as a Possible Mechanism of Action of Guanethidine (SU 5864), a New Hypotensive Agent. (25702)

ROSEMARY CASS,* RONALD KUNTZMAN AND BERNARD B. BRODIE
Lab. of Chemical Pharmacology, Nat. Heart Inst., U. S. Dept. of H. E. W., Bethesda, Md.

A recent report(1) described pharmacological properties of 2-(octahydro-1-azocinyl)-ethyl-guanidine sulfate (Su-5864, guanethidine). This compound produced a variety of sympatholytic effects of prolonged duration, including lowering of arterial pressure. The drug acted by making the peripheral sympathetic system unresponsive to stimuli. The unresponsiveness could not be attributed to interference wtih conduction of nerve impulses or transmission across ganglia, or to blocking the action of norepinephrine. It was suggested that guanethidine might interfere with release and/or normal distribution subsequent to release of the neurohumoral transmitter at the sympathetic neuromuscular junction. The present paper shows that guanethidine decreases level of norepinephrine in heart and spleen without lowering norepinephrine level in the brain.

Methods and Materials. Rabbits, New Zealand white, weighing about 2 kg, and mongrel cats, weighing 3 to 4 kg, were used in these studies. Rabbits were given guanethidine (Su-5864)[†] in a single dose of 12.5 mg/kg intravenously; cats were given a single dose of 15 mg/kg subcutaneously. Immediately after

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[*] Present address: Dept. of Pharmacology, School of Pharmacy, University of London, London, England.

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TABLE I. Norepinephrine (NE) Concentration in Rabbit Heart Various Times after Administration of Su-5864 (12.5 mg/kg I.V.).

Time, hr	No. of animals	Conc. of norepinephrine, $\mu g/g$
0	11	1.78 (1.31-2.31)
1	4	1.17 (.91-1.34)
2	2	.69 (.59- .78)
4	6	.39 (.35- .42)
18	3	.27 (.13- .48)
48	2	.85 (.80- .90)
72	2	1.13 (1.11-1.15)
120	2	1.35 (1.29-1.42)
168	2	1.98 (1.82-2.14)

Figures in parentheses represent range of values.

injection some rabbits showed signs of transient apnea and prostration. At various times after drug administration the animals were killed by intravenous injection of air or by chloroform. The tissues were analyzed for serotonin and norepinephrine by methods previously described(2,3).

Results. Norepinephrine content of rabbit heart declined progressively for about 4 hours after guanethidine administration, reaching a level 15% that of normal (Table I). The norepinephrine level remained at this low value for at least 18 hours, had partially recovered in 48 hours, and reached normal value in about 7 days. The norepinephrine level in spleen also declined about 60% in 18 hours. However, norepinephrine levels in brain or adrenal medulla were not affected. In addition, there was no decrease in level of serotonin in brain over a period of 18 hours.

Guanethidine in the cat caused a decrease in heart norepinephrine level by about 75% in 24 hours but did not lower norepinephrine level in brain or adrenal medulla.

Discussion. Guanethidine, like reserpine, produces a decline in content of tissue norepinephrine in the rabbit and cat. The catecholamine depletion observed after guanethidine (12.5 mg/kg) is slower however than after reserpine(4). The effect on norepinephrine could be due either to release of the amine or to blocking of its synthesis. Both of these possibilities are now being investigated.

The failure of guanethidine to lower content of brain norepinephrine is not surprising as the drug is unlikely to cross the blood-brain barrier readily, due to its extremely low lipid solubility (compound not extracted from buffer pH 7.4 into chloroform).

The data presented here suggest that guanethidine lowers blood pressure by producing chemical sympathectomy through depleting norepinephrine from peripheral nerve endings.

Summary. Guanethidine (Su-5864), a new hypotensive agent, depletes the norepinephrine level in the heart of rabbits and cats. It is suggested that guanethidine lowers blood pressure by producing chemical sympathectomy through depletion of norepinephrine from peripheral nerve endings.

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A Simple Method for Concentration of Live and Formaldehyde-Inactivated Poliovirus. (25703)

NATHAN GROSSOWICZ, ALBERT MERCADO AND NATAN GOLDBLUM
Dept. of Bacteriology, Hebrew University-Hadassah Medical School, Jerusalem, and
Virus Laboratory, Ministry of Health, Tel Aviv-Yaffo, Israel

Salk type vaccine has been widely used for immunization against poliomyelitis. Since antigenicity of currently used vaccine preparations is not uniformly satisfactory(1), concentration of poliovirus suspensions to be transformed into vaccine or of formalinized vaccines may provide considerably more potent immunizing agents. Several methods for concentration of poliovirus preparations have been described. One is based on precipitation with methanol and further purification of the virus-containing material by elution, ultracentrifugation and enzyme treatment(2,3); others use chromatography on cellulose ion-exchange columns(4,5), and on calcium or aluminium phosphate gels(6,7). These procedures, mainly aimed at obtaining pure virus preparations, are not easily adaptable to large scale vaccine concentration. This communication deals with a simple single-step method for concentration and partial purification of poliovirus preparations by precipitation with salts of various bivalent cations. Both active virus and formalinized poliomyelitis vaccine can be concentrated by this method.

Materials and methods. Poliovirus suspensions were prepared in monkey kidney cell monolayers using medium 199. Crude virus preparations were passed through Seitz clarifying pads, grades K3 and K7, then filtered through Seitz sterilizing (STl) pads. These Seitz filtrates were used for precipitation with salts. Formalinized monovalent vaccine pools were prepared according to methods described earlier(8,1). Most of the work was carried out using cobalt chloride as precipitating agent. Salts of Ca^{++}, Ba^{++} and Mn^{++} were also employed. The procedure with $CoCl_2$ is outlined briefly as follows: A Seitz-filtered virus suspension is alkalinized to pH 8.8-9.0 by careful addition of NaOH (0.1 M to 2 M alkali according to initial volume processed). Cobalt chloride, 1 M solution, is added with stirring to final concentration of approximately 1 mg Co^{++}/ml virus suspension. A flocculate appears almost immediately and settles down in a few minutes. Complete clarification of the supernatant fluid is obtained after standing about 1 hour at 4°C, or by centrifugation 2-3 minutes at about 1000 rpm. The supernatant is decanted, and the precipitate (bluish in color) is taken up in small volume of McIlvaine's buffer pH 3.0 (about 4 vol. of 0.1 M acetic acid and 1 vol. of 0.2 M disodium phosphate) and dialyzed several hours against approximately 100 vol. of buffer. Degree of concentration of live virus is determined by titration in monkey kidney cell culture tubes using 6 to 8 tubes/log dilution. Concentration of formalinized vaccine is carried out similarly. However, a slightly higher initial pH (9-9.3) is required for satisfactory precipitation. Clarification of precipitate is achieved by dilution 1:4 with citric acid/phosphate buffer, pH 4.2-4.4. Partial purification and removal of Co^{++} is obtained by dialysis against citric acid buffer, pH 5.0-6.0. Degree of concentration of the antigen is determined by potency testing in guinea-pigs using the method of Gard *et al.* (9) or in chicks according to De Somer *et al.* (10) or by *in vitro* tests measuring combining capacity with neutralizing antibody(11,12).

Results reported here were obtained by using cobalt as precipitating agent in the form of 1 M $CoCl_2$ solution. However, salts of Ca^{++}, Ba^{++} and Mn^{++} gave essentially similar results in preliminary trials. Table I shows results obtained with two 1000 ml samples of type 2 poliovirus suspensions and Table II illustrates concentration of type 1 poliovirus vaccine. In both, $CoCl_2$ was used for precipitation. The method enabled concentration of live poliovirus about a thousand-fold, and that of monovalent vaccine pools, over one-hundred-fold, with only negligible losses of virus or antigen. So far, live virus preparations of types 2 (MEF-1) and 3 (Saukett) and monovalent vaccine pools types 1 (Brunhilde) and 3 were concentrated. Work is in progress to assess degree of purification of these concentrated preparations as well as the mechanism of reaction. A detailed de-

TABLE I. Concentration of Type 2 Poliovirus Suspensions Using Cobalt Chloride as Precipitating Agent.

		Before conc.	After conc.	Degree of conc.	% recovery
Sample 1	Vol (ml)	1000	1.0	1000	
	Infectivity titer*	7.4	10.3	Ca. 1000	Ca. 99
" 2	Vol (ml)	1000	5.0	200	
	Infectivity titer*	7.3	9.6	Ca. 200	Ca. 99

* Expressed as negative logarithm of 50% tissue culture infective dose end point.

TABLE II. Concentration of Type 1 Monovalent Poliomyelitis Vaccine by Cobalt Chloride.

	Before conc.	After conc.	Degree of conc.	% recovery
Vol (ml)	1000	7.0	Ca. 140	
Potency in guinea-pigs (extinction point)	2.0	4.2a*	Ca. 140	100
		3.3b*	20	15

* Expressed as negative logarithm of 50% tissue culture infective dose end point. (a) and (b) represent results of 2 separate assays of potency on same concentrate; (a) potency determined shortly after concentration; (b) potency determined after concentrated undialyzed material has been stored at 4°C for 3 mo.

scription of various methods and results will be published elsewhere.

Comments. The method may have various advantages because of its simplicity, and the results obtained seem very promising. The possibilities in use of this procedure may be as follows: a) reduction of large volumes of virus suspensions used in preparation of formalinized vaccine on an industrial scale; b) preparation of antigens for use in complement fixation or other serological reactions; c) concentration of antigen in vaccine preparations of low antigenicity and d) if concentration is accompanied by purification, removal from the vaccine of potentially harmful substances originating in destroyed monkey kidney cells. All these will have to be tested experimentally. When processing larger volumes of material, continuous flow or Sharpless centrifugation may be used instead of batch treatment. It appears that this method can be adapted with ease to concentration of other viruses grown on tissue culture.

Summary. A simple and efficient method for concentration of poliovirus preparations is described. Bivalent cations, and particularly cobalt chloride, are used as precipitating agents, and the precipitate is resuspended with acid and/or by dialysis. Concentration of both live virus preparations as well as formalinized vaccine (Salk type) may be obtained in this way. The method permits concentration of live poliovirus about one thousand-fold and that of vaccine over one-hundred-fold, with negligible losses of virus or antigen.

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Estradiol-17β and Progesterone in Ovaries of Starfish (*Pisaster ochraceous*).* (25704)

CHARLES R. BOTTICELLI, FREDERICK L. HISAW, JR.,† AND HERBERT H. WOTIZ‡
(Introduced by Frederick L. Hisaw)
Biological Labs, Harvard University, Mass.; Dept of Biochemistry, Boston University School of Medicine; and University of Washington, Friday Harbor Labs, Seattle, Wash.

Extracts that produce estrogenic reactions in mammals have been obtained from representatives of several Phyla of invertebrates (1) but the chemical identity of substances responsible for these effects is unknown. Estrogenic preparations have been obtained from gonads of starfish by Steidle(2), Donahue and Jennings(3), and more recently by Hagerman *et al.*(4). That such active agents are chemically related to steroid hormones of vertebrates is, of course, a possibility and their presence may be of some evolutionary significance. The present report presents results of effort to isolate estrogens and progesterone from ovarian tissue of starfish (*Pisaster ochraceous*) by methods commonly employed for obtaining these hormones from mammalian material.

Procedures. Starfish ovaries (6552 g) were collected and stored in approximately twice their volume of acetone. At time of extraction the acetone was poured off, ovarian tissue homogenized and reextracted with acetone. The combined acetone extracts were taken to dryness by gentle stream of warm air passing over the solution in large evaporating dishes. The oily residue was dissolved in sufficient 70% ethanol and extracted 3 times with ligroin (bp. 30-60°C). The ethanolic fraction was diluted with water containing 35% alcohol and subsequently washed with ligroin (3x). The alcoholic solution, bioassayed by the Astwood method(5), showed presence of estrogenic material in very small amount. The ethanolic fraction was then dried *in vacuo* and the residue partitioned countercurrently between 70% methanol and a 50-50 mixture of chloroform and carbon tetrachloride with 29 transfers. Five fractions were collected by combining tubes 0-2, 3-9, 10-19, 20-25, and 26-29, and dried *in vacuo*. The residue for each group of tubes was dissolved in 10 ml of methanol and 1 ml aliquot was taken from each for bioassay of estrogenic potency. Propylene glycol (1 cc) was added to each aliquot and the alcohol evaporated. Rats from inbred strain were 24 or 25 days old and weighed 54 to 60 g. Two animals were used to test material from each set of tubes. They were given 0.4 cc of propylene glycol solution in single subcutaneous dose and uteri removed 6 hours later and weighed.

The *results* (Table I), indicated estrogenic activity only in material obtained from tubes 3-9, and 10-19. Therefore, these 2 fractions were recombined and again partitioned in the same system with 29 transfers. The tubes of this second run were combined in 3 groups, 0-8, 9-20, and 21-29 and were processed as before. Results of the bioassay (Table II) show activity in fractions 0-8, and 9-20. It seems quite probable that estrogenic activity in tubes 3-9 of the first run and subsequently in 0-8 of the second run was caused by the tailing of estradiol, a supposition subsequently borne out by chromatographic evidence.

Fractions 0-8 and 10-19 were each divided into 3 aliquot portions and subjected to the following 3 paper chromatographic systems, at room temperature: (1) Benzene-formamide (1:1) for 22 to 24 hours; (2) toluene-propy-

TABLE I. Results of Bioassay from First Countercurrent Separation.

Fraction	Uterine wet wt, mg	% increase over control
Controls*	22.4	
0- 2	21.9	.0
3- 9	26.2	15.9
10-19	28.8	28.5
20-25	22.4	.0
25-29	22.0	.0

* 30 animals.

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† Department of Zoology, Oregon State College, Corvallis, Oregon.

‡ Senior Research Fellow, U.S.P.H.S.

TABLE II. Bioassays for Second Countercurrent Distribution.

Fraction	Uterine wet wt, mg	% increase over control
Controls*	22.4	
0- 8	23.4	4.5
9-20	25.7	14.7
21-29	22.6	.0

* 30 animals.

lene glycol (1:1) for 6 hours, and (3) ligroin-propylene glycol (1:1) for 64 hours. The unknown fraction in each of these systems was applied to one of 4 strips, estrone to another, estradiol to a third and a mixture of estrone and estradiol to the fourth. The 4 strips in each system were run simultaneously. The 3 strips treated with authentic estrogens were stained with ferric chloride-ferricyanide stain. Areas on experimental strips corresponding to the standards were cut and eluted 5 times with 10 ml portions of absolute ethanol. Similar elutions also were made of the remaining paper from experimental strip. Alcoholic eluates were evaporated in part, transferred to small vials, and reduced to dryness. Propylene glycol (0.5 cc) was added to each vial and 0.2 cc was injected in single subcutaneous dose into each of two 24-25 day old rats. Results of these bioassays (Table III) indicate presence of estradiol-17β, while test fractions corresponding to authentic estrone gave uniformly negative results. Also, the eluates of the paper outside the test areas gave negative responses.

While these results strongly indicate presence of estradiol-17β in ovarian tissue of the starfish, they also emphasize its small amount. In view of this, and assuming that estradiol can be converted to estrone in the starfish, it seems quite probable that estrone also might have been present but in a quantity insufficient for detection by our methods. The bioassay used has been very carefully standardized on a strain of inbred rats, in which minimal dose of estradiol that induces a 33% increase in uterine weight in 6 hours is 0.025 μg and estrone 0.45 μg(5,6). It has been estimated, for comparison, that ripe ovaries of the starfish contain between 0.04 μg and 0.1 μg of free estradiol-17β/kg of fresh tissue.

The brown oily residue obtained on evaporation of the ligroin fractions was used in procedures for isolation of progesterone. This material was first applied in benzene and ligroin (30 ml) to an alumina (Merck) column (24 mm x 170 mm) and eluted with petroleum ether, benzene and ethyl acetate, primarily for cleaning the preparation. Seven eluates were obtained, of which the third (petroleum ether, 100 ml) and the seventh (ethyl acetate, 300 ml) gave positive reactions when tested by Hooker-Forbes method(7).

Eluates 1-4 and 5-7 were combined, evaporated to dryness and applied in benzene to 2 separate aluminum (100 g Woelm, neutral activity grade II) columns (24 mm x 170 mm). A similar column was also treated with authentic progesterone. The columns were eluted with 100 ml of ligroin plus graded amounts of benzene, benzene plus chloroform, and chloroform plus methanol. Eluates were tested by Hooker-Forbes method, and positive reactions were obtained in fractions from both experimental columns corresponding to those containing authentic progesterone from the control column. The only difference was

TABLE III. Bioassay of Eluted Paper Strips from 3 Different Chromatographic Systems.

	Countercurrent fractions 0-8 Chromatography*				Countercurrent fractions 9-20 Chromatography*			
	Estrone		Estradiol		Estrone		Estradiol	
Chromatographic systems	Uterine wet wt, mg	% increase	Uterine wet wt, mg	% increase	Uterine wet wt, mg	% increase	Uterine wet wt, mg	% increase
Benzene-formamide	22.4	0	25.5	13.8	21.0	0	26.0	16.1
Toluene-propylene glycol	22.5	0	24.2	8.1	21.4	0	24.3	8.5
Ligroin-propylene	21.1	0	24.1	6.7	22.7	0	26.6	18.7

* Areas of test strips corresponding to authentic estrone and estradiol-17β. Uterine weights are avg for 2 animals in each experimental test. Avg uterine wet wt for 30 untreated control animals was 22.4 ± 0.3 mg.

a somewhat wider spread of activity in fractions from experimental columns than in those of the control. However, the active substance was present in such small amount that further identification was not attempted.

Our results strongly indicate that ovaries of starfish (*Pisaster ochraceous*) at time of spawning contain estradiol-17β. Estrone was not found. However, as concentration of estradiol was estimated to be approximately 0.04 to 0.1 μg/kg of fresh tissue, the amount of estrone that could have been derived as a metabolite from this source would probably be too small for detection by methods employed.

Discussion. Evidence for presence of progesterone, though not as convincing as that for estradiol, is, nevertheless, significant. The chromatographic agreement between authentic progesterone and the substance capable of evoking Hooker-Forbes response may not be conclusive proof of their identity but it is a definite possibility. The amount of active material obtained was too small for a more thorough characterization so absolute identification will require further study.

The significance of these observations lies in the possibility that ovarian, steroid hormones of vertebrates may also be present in gonads of invertebrates and raises questions regarding physiological and evolutionary importance.

Summary. Ovaries of starfish (*Pisaster ochraceous*) were examined for sex steroids. A low level of estrogenic activity, approximately 0.1 μg or less/kg of ovarian tissue, was determined by the Astwood 6 hour assay method. The biologically active material was identified as estradiol 17β by countercurrent distribution, paper chromatography and bioassay. Presence of progesterone was tentatively identified by methods of column chromatography and Hooker-Forbes assay.

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